The role of DNA methyltransferases in plant genomic imprinting

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Abstract

Genomic imprinting is the epigenetic modification of loci, primarily by DNA methylation, which results in parent-of-origin-specific monoallelic expression of a small subset of genes. In plants, imprinting occurs during endosperm development and a balance of maternally- and paternally-expressed imprinted genes is essential for normal seed development. Dependence on DNA methylation for imprinting highlights the potential to manipulate seed development, and consequently seed size, by altering DNA methyltransferase activity. DNA METHYLTRANSFERASE 1 (MET1) is the primary plant maintenance DNA methyltransferase and plays a significant role in imprinting. However, no evaluation of the potential role for other MET1 family members in genomic imprinting has been reported. The current model for the control of imprinting in plants suggests that maintenance DNA methyltransferases are required throughout development, yet the tissue-specific requirement of these enzymes is unconfirmed as analysis has relied solely on constitutive DNA methyltransferase mutants. To address these problems and to evaluate the potential to alter seed size, the work reported in this thesis investigated the potential involvement of putative maintenance DNA methyltransferases MET2a, MET2b and MET3 and the tissue-specific role of MET1 in imprinting. Imprinting was not significantly altered in *met2a-1*, *met2b-1* and *met3-1* mutants, indicating that MET1 is the sole DNA methyltransferase required for imprinting. Transcriptional analysis suggested *MET1* is expressed throughout floral organ development and in the male and female gametophyte generation indicating that MET1 is potentially available to maintain imprinting-dependent methylation in these tissues. Tools to suppress *MET1* tissue-specifically were developed to investigate the tissue-specific requirement of MET1 for imprinting. Analysis indicates that such tools could also be used to alter seed size by manipulating imprinting in commercially important species. Further work is needed to validate this approach.

Abbreviations and acronyms

AP3	APETALA3
as	antisense
BAH	bromo-adjacent homology
BAP	benzylaminopurine
bp	base pair/s
BASTA	glufosinate ammonium
CaMV	cauliflower mosaic virus
Col-0	Colmbia-0
DAP	days after pollination
DDM1	decreased DNA methylation 1
DNMT1	DNA methyltransferase 1
DMR	differentially methylation region
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetetic acid
F1	first filial generation produced by crossing two parental lines
G1	generation 1
GFP	green fluorescent protein
GUS	ß-glucuronidase
HAP	hours after pollination
IR	inverted repeat
Ka	nonsynonymous substitutions per nonsynonymous site
KanR	kanamycin resistance
Kb	kilo base pairs
$K_3Fe(CN)_6$	potassium ferricyanide
$K_4Fe(CN)_6.3H20$	potassium ferrocyanide
Ks	synonymous substitutions per synonymous site
LB	left Border
MET1	DNA METHYLTRANSFERASE 1
MET2A	DNA METHYLTRANSFERASE 2a
MET2B	DNA METHYLTRANSFERASE 2b
MET3	DNA METHYLTRANSFERASE 3
$MgCl_2$	magnesium chloride
min	minutes
mRNA	messenger RNA
NAA	naphthalene acetic acid
NASC	Nottingham Arabidopsis Stock Centre
NLS	nuclear localisation signal
Nos	nopaline synthase
PCR	polymerase chain reaction
PgC	polycomb group proteins
RB	right border
RFTS	replication foci targeting sequence
rpm	revolutions per minute
RNA	ribonucleic acid
RNAi	RNA interference
RT PCR	reverse transcription PCR
SAM	shoot apical meristem

S.E.M.	standard error of the mean
SHP2	SHATTERPROOF2
SDS	sodium dodecyl sulphate
TAC	transformation-competent artificial chromosome
TAE	Tris-acetate-EDTA
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
WS	Wassileskija
WT	wild type

Contents

Chapter 1 - *Introduction and aims*

1.1 *Why research seed development?*

Seed production is fundamental for global food and feed security and has huge economic importance worldwide. This section briefly considers how enhancing our understanding of seed development may play a key role in securing adequate seed production throughout the 21st century and beyond.

Between 1959 and 1999 world population doubled from 3 billion to 6 billion and is estimated to reach 9 billion by 2042 (IDB, 2007). An increasing population imposes continuous and increasing pressure on agriculture to increase yields. Malnutrition already poses an acute problem in developing countries. More than 0.8 billion people have too little to eat to meet their daily energy needs and in developing countries more than a quarter of children under 5 are malnourished (FAO, 2007). Cereal grains provide the most important staple food source. Three cereals crops, namely rice, wheat and maize, provide 50 % of the world's average diet and make up more than two thirds of food energy intake (FAO, 2007). Consequently, there is an emphasis to increase the yield of cereal varieties.

Between the 1940s and the 1960s the Green Revolution helped avert large scale famine by enabling food production to keep pace with population growth. During this period cereal production was dramatically increased due to the development and adoption of high-yielding semi-dwarf varieties of wheat and rice (Sakamoto, T. and Matsuoka, M. 2004). Today it is believed that biotechnology can play a decisive role in increasing productivity and improving food security (Ozor, N. and Igbokwe, E. M. 2007). Many countries already benefit from yield increases resulting from the adoption of genetically modified crops species. For example, on-farm trails in India revealed a 60% increase in yield from Bt Cotton compared with non-Bt equivalent varieties (Qaim, M. and Zilberman, D. 2003).

The economic benefits of increasing seed production are also significant. In 2006 the value of the cereal produced in the UK alone was estimated at £1.6 billion and at present the UK cereal production constitutes only 1% of the global market (DEFRA, 2007, FAO, 2007). During 2007 wheat prices in the UK increased by more than 100% (DEFRA, price series for cereals 2007), increasing seed production by genetic manipulation therefore has the potential to bring important economic benefits.

It is believed that an in depth understanding of the developmental biology of the organ, or trait, to be altered is essential to achieve the full potential from genetic manipulation. The work described in this thesis contributes to a greater understanding of seed development in two model species; *Arabidopsis thaliana* and tobacco. Additionally this thesis describes the analysis of tools to manipulate seed development for yield improvement. Such research is essential to fulfil the potential future benefits of plant genetic manipulation.

1.2 *Seed development in flowering plants*

This section reviews the life history of flowering plants, the function of the endosperm during seed development and how seed size can be altered.

1.2.1 *The alternation of generations and double fertilization*

The life history of plants involves the alternation between diploid sporophyte and haploid gametophyte generations In flowering plants the sporophyte generation constitutes the majority of the life cycle and the gametophyte is greatly reduced (**Figure 1.1**).

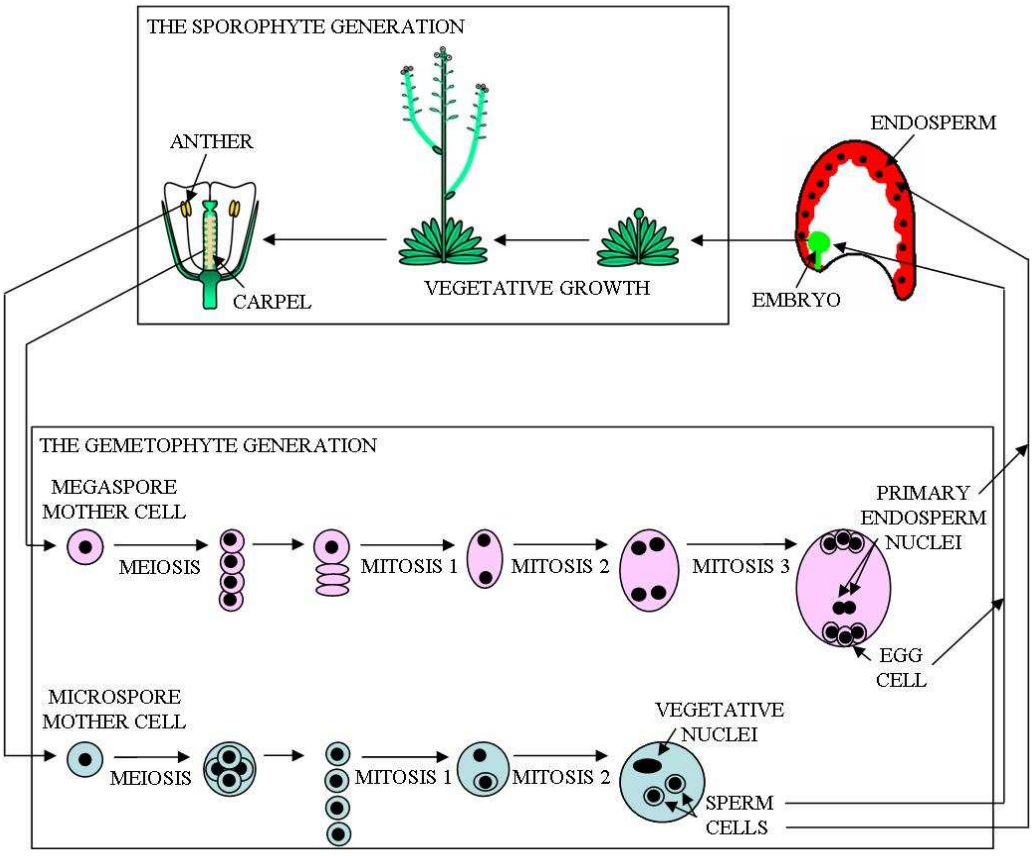


Figure 1.1 The life history of flowering plants: the alternation of generations, male and female gametogenesis and double fertilization.

The male gametophyte, or pollen grain, is formed within the anther of the sporophyte. Diploid microspore mother cells undergo meiosis to produce tetrads of haploid microspores. The nucleus of each microspore divides mitotically to produce a vegetative and a generative nucleus. The generative nucleus undergoes a second mitotic division to form two sperm cells; the male gametes. The sperm cells are harboured within the cytoplasm of the vegetative cell. Male gametogenesis is reviewed further by McCormick, S. (2004).

The female gametophyte, or embryo sac, is formed within the ovary which in turn develops within the carpel of the sporophyte. A diploid megaspore mother cell undergoes meiosis to produce a tetrad of haploid megaspores. Commonly, three megaspores degenerate and the surviving one develops into the female gametophyte. The female gametophyte can vary in form but the majority of flowering plants produce a *polygonum*-type gametophyte. This consists of seven-cells and eight-nuclei, derived from three mitotic divisions of the megaspore nucleus. Cellularisation of the embryo sac produces three antipodal cells that reside at the chalazal pole, two synergid cells and an egg cell at the micropylar pole and a central cell containing two polar nuclei that is positioned between the two poles. Frequently, the polar nuclei fuse before fertilisation. The egg and central cells constitute the two female gametes. Female gametogenesis is reviewed further by Christensen et al (1997).

Seed development in flowering plants is initiated by double fertilization. One haploid sperm fuses with the haploid egg to produce the diploid embryo and the second haploid sperm fuses with the diploid central to form the triploid endosperm (**Figure 1.1**). The embryo has a maternal: paternal parental genomic ratio of 1:1 (1m:1p) whereas the ratio in the endosperm is 2m:1p.

1.2.2 *Endosperm development and function*

The endosperm is a nutrient sink that is supplied by the seed parent and nourishes the embryo during embryogenesis or germination (Lopes, M. A. and Larkins, B. A. 1993). Cereals produce a persistent endosperm which constitutes the bulk of a mature cereal grain and provides a nutrient store to fuel germination. In contrast, dicotyledonous plants like *Arabidopsis* produce a transient endosperm that is largely consumed during embryogenesis. Consequently, the bulk of a mature *Arabidopsis* seed consists of the

embryo cotyledons which provide a nutrient store to fuel germination. Despite these differences, endosperm development in *Arabidopsis* and cereals has significant similarities and thus *Arabidopsis* is considered a useful model for the analysis of seed development in a wide range of plant species.

As with most angiosperms, *Arabidopsis* and cereals produce nuclear-type endosperm which is characterised by a phase of syncytical division without cytokinesis followed by cellularization (Oslen, O. 2004). In *Arabidopsis*, the endosperm syncytium forms three regions, the micropylar endosperm, the peripheral endosperm and the chalazal endosperm (Brown, R. C. et al. 1999). When the embryo reaches the heart stage of development cellularization of the endosperm is initiated at the micropylar pole and moves in a wave toward the central chamber of the embryo sac. The chalazal pole remains syncytial until the late stages of seed maturity (Brown, R. C. et al. 1999).

Similar endosperm regions are found in both *Arabidopsis* and cereals (**Figure 1.2**) suggesting that the function of these regions is conserved across the angiosperms (Costa, L. M. et al. 2004). The micropylar endosperm, or embryo surrounding region in cereals, forms adjacent to the embryo. In maize, this region expresses putative signalling peptides consistent with a role in embryo/endosperm communication (Bonello, J. F. et al. 2002). The peripheral endosperm, or aleurone in cereals, forms an epidermal layer inside the seed cavity. This layer is involved in mobilizing starch and protein during germination (Oslen, O. 2004). The chalazal endosperm, or basal endosperm transfer region in cereals, forms close to maternal vascular tissue. In cereals, this region is involved in the uptake of nutrients for grain filling (Thompson, R. D. et al. 2001). Nutrients transported through the vascular system are taken up by transfer cells of the nucellus, deposited in the apoplasm and then taken up by transfer cells in the basal endosperm transfer region and transferred for storage (Patrick, J. W. and Offler, C. E. 2001). Due their position on top of the nucellus proliferating tissue and with close proximity to maternal vascular tissue, chalazal endosperm cysts may also function in nutrient up take in *Arabidopsis* (Brown, R. C. et al. 1999). Cereals also have a starchy endosperm region which constitutes the bulk nutrient reserve in mature grains. As suggested above, the cotyledons provide the bulk nutrient reserve in mature *Arabidopsis* seed.

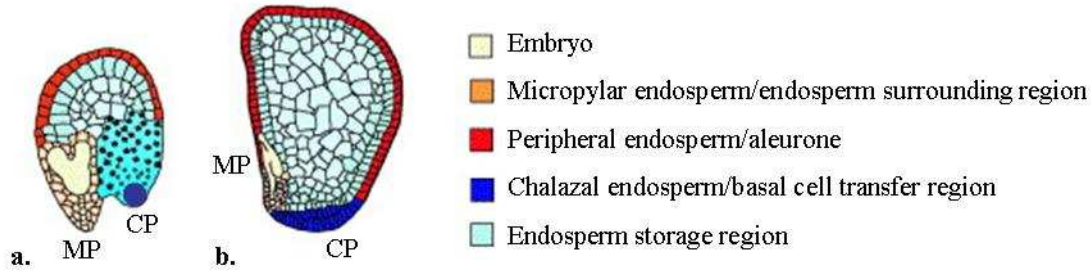


Figure 1.2 Endosperm regions in an immature *Arabidopsis* seed and a rice grain.
a. An immature *Arabidopsis* seed at cellularization. As the seed matures the embryo will engulf the endosperm storage region and the embryo cotyledons will fill the seed cavity**.** **b.** An immature rice grain. As the grain matures, protein and starch will accumulate in the endosperm storage region. This will be utilised at germination. MP and CP mark the micropylar pole and chalazal pole respectively. These images were adapted from (Costa, L. M. et al. 2004).

1.2.3 *Altering seed development*

One approach to increase cereal yield is to increase grain size. Since the beginning of agriculture, grain size has been subject to selection and breeding, consequently most of the grains consumed today are far larger than their wild relatives (Sundaresan, V. 2005). More recently, analysis of mutants with altered seed size has increased our understanding of how seed development is controlled and has thus highlighted aspects of development which could be selectively manipulated to alter seed size. For example, the *auxin response factor 2* mutant has increased seed weight as a consequence of increased integument cell division (Schruff, M. C. et al. 2006). This indicates that seed size could be manipulated by altering cell division. Additionally, endosperm proliferation has been identified as an important factor in determining seed size; mature seed size is positively correlated with endosperm proliferation, even in species with transient endosperm (Scott, R. J. et al. 1998). The relationship between endosperm proliferation and seed size has been reiterated recently by the identification of a new class of small seed mutants. *haiku1*, *haiku2* and *mini3* have reduced seed size as a result of precocious endosperm cellularization and reduced endosperm proliferation (Garcia, D. et al. 2003, Luo, M. et al. 2005). One factor influencing the extent of endosperm proliferation is genomic imprinting, an epigenetic phenomenon inferring parent-of-origin-specific gene expression (Scott, R. J. et al. 1998).

The work described in thesis investigates how seed size can be altered by manipulating genomic imprinting. The following sections review how genomic imprinting was

identified, the importance of genomic imprinting for development, and the current understanding of how imprinting is controlled.

1.3 *Genomic Imprinting*

Genomic imprinting is the epigenetic modification of loci which results in parent-of-origin-specific expression (Crouse, H. V. 1960). In the zygote of a species exhibiting genomic imprinting, a small subset of genes are expressed from only their maternally-inherited allele whilst others expressed from only their paternally-inherited allele. Imprinting has been more intensively studied in mammals than in plants. However, despite comparative paucity in our understanding of this phenomenon in plants, some interesting similarities between the imprinting systems of plants and mammals are becoming apparent. In recognition of this, it is proposed that a greater understanding of imprinting in plants can be gained from studying mammals, and vice-versa. In this chapter, the imprinting systems of both mammals and plants are reviewed.

1.3.1 *Genomic imprinting and the parental conflict theory*

The most widely accepted explanation for the evolution of genomic imprinting is the parental conflict or kinship theory which proposes that genomic imprinting evolved to control the transcription of loci whose expression has a fitness consequence for kin (Haig, D. and Westoby, M. 1989, Haig, D. and Westoby, M. 1991 and Haig, D. 2000). For instance, in polyandrous species, paternally-derived alleles in offspring are likely to be less closely related to those in siblings from the same mother than maternally derived alleles. Paternally-derived alleles are therefore selected to extract maternal resources at the expense of their siblings or order to achieve the greatest fitness benefit. In contract, maternally-derived alleles will achieve the greatest fitness benefit if maternal resources are distributed between offspring. Similarly, a mother is equally related to her offspring and therefore will receive the greatest fitness benefit when distributing her resources equally between them. In contrast, a father will receive the greatest fitness advantage if the mother concentrates her resources on *his* offspring. Thus the parental conflict theory proposes that genomic imprinting evolved as a consequence of conflict between maternally- and paternally-inherited genomes over resource allocation from mother to offspring.

The theory predicts that imprinting can occur only in species which exhibit a 'placental habit' (Haig, D. and Westoby, M. 1991); in others words, in species which rely on the direct transfer of resources from mother to offspring '*in utero*'. In agreement of these hypothesis, there is little evidence that imprinting occurs in fish, reptile, amphibian and bird species, which tend to leave their offspring to their own devices post conception (Killian, J. K. et al. 2000). Additionally, the theory predicts that selection will favour the expression of paternally-inherited alleles that promote resource transfer from mother to offspring and the expression of maternally-inherited alleles that restrict resource transfer. In agreement, many imprinted genes fit this trend (See **1.3.2** and **1.3.3**).

1.3.2 *Genomic imprinting in mammals*

Genomic imprinting first became apparent in mammals as a result of nuclear transplantation experiments in mouse. One-cell stage embryos containing either two male or two female pronuclei failed to complete normal embryogenesis demonstrating that the maternally- and paternally-inherited genomes are not equivalent and both genomes are required for normal development (McGrath, J. and Solter, D. 1984, Surani, M. A. H. et al. 1984). More than eighty imprinted genes have now been identified in mouse and this number is increasing steadily. The Mammalian Genetic Unit, have compiled a database of all mammalian imprinted genes and this regularly updated (Beechey C.V. et al. 2007). The database shows that a substantial proportion of the imprinted genes are involved in foetal growth. In support of the parental conflict theory, paternally-expressed genes generally act to increase resource transfer from mother to offspring, whereas maternally-expressed genes act to reduce resource transfer.

Many imprinted genes are expressed in the placenta and foetus and are hypothesised to control both the foetal demand and placental supply of material nutrients (Reik, W. et al. 2003). In agreement with this hypothesis, several imprinted gene mutants have altered placenta and foetal development. For example, in mutants for the paternally-expressed growth factor Igf2, placenta and foetal weights are reduced by 50% and 40% respectively, whereas mutants for the maternally-expressed IGFII receptor Igf2r, placenta and foetal weights are increased by 30% and 40% respectively (Fowden, A. L. et al. 2006).

Interestingly, imprinting continues during postnatal development in mammals. As mothers continue to transfer resources to their offspring, most obviously via suckling, postnatal

imprinting can also be explained by the parental conflict theory (Constancia, M. et al. 2004). Several disorders in humans affect growth and development after birth and are correlated with the absence or misexpression of imprinted genes. One example is Prader-Willi syndrome which is caused by mutations in imprinted genes on the paternally-inherited chromosome 15. Children born with Prader-Willi syndrome show little interest in suckling during the first months of their life (Holm, V. A. et al. 1993). This indicates that paternally-inherited genes may act to encourage suckling, and thus postnatal resource transfer from mother to offspring (Constancia, M. et al. 2004).

1.3.3 *Genomic imprinting in flowering plants*

It was hypothesised that genomic imprinting occurs in the endosperm of flowering plants because the endosperm functions to transfer resources from the seed parent to the embryo (Haig, D. and Westoby, M. 1989, Haig, D. and Westoby, M. 1991). Substantial evidence supports this hypothesis and the occurrence of imprinting in the endosperm is widely accepted. Imprinting has not been reported in the embryo or in the mature sporophyte, presumably because the embryo does not obtain resources directly from the seed parent and has no access to maternal resources once the seed is shed the plant. This section reviews how imprinting in the endosperm was discovered and the imprinted genes identified in plants to date.

1.3.3.1 *Evidence of genomic imprinting in the endosperm*

The first indication of genomic imprinting in plants came from the apparent ploidy barrier preventing normal seed development from interploidy crosses, both within a species and between species. Ploidy barriers are reported for many crosses and several examples are detailed in Haig, D. and Westoby, M. (1991). Original hypotheses to explain this phenomenon were based on the assumption that parentally-inherited genomes are equivalent. Many hypotheses proposed that incompatibility of crosses resulted from an imbalance in ploidy ratios of various components of the seed, for example a disturbance of the 2:3:2 ploidy ratio of maternal tissue to endosperm to embryo (Müntzing, A. 1933), the 3:2 ploidy ratio of endosperm to embryo (Watkins, A. E. 1932) or the 2:3 ploidy ratio of maternal tissue to endosperm (Valentine, D. H. 1954).

Kermicle, J.L. (1970) produced the first report indicating that maternally- and paternally-inherited genomes are not equivalent and are therefore subject to genomic imprinting.

Kermicle studied the genetics of the *r* locus in maize and discovered that maternal inheritance of the *R* allele results in solidly coloured kernels are produced, whereas paternal inheritance produces mottled kernels. This was shown to be independent of dosage affects and it was concluded that the action of the R allele is dependent on its gametophytic passage prior to fertilization.

Following the realization that the maternally- and paternally-inherited genes are not equivalent, a number of hypotheses suggested that the ploidy barrier was a result of an imbalance in the relative generic values of parental genomes. For example Johnston et al (1980) proposed that each species should be assigned a specific Endosperm Balance Number (EBN) based on their crossing behaviour with a standard species. It was suggested that the success of a cross was dependent on achieving a 2:1 maternal: paternal ratio of the EBNs of parental plants (Johnston, S. A. et al. 1980).

Lin, B. (1984) produced the first genetic evidence that a 2m:1p genomic ratio in endosperm is critical for normal embryogenesis and seed development in plants. Lin worked with the maize *indeterminate gametophyte* (*ig*) mutants in which the coding region of a *LATERAL ORGAN BOUNDARIES* (*LOB*) domain gene is disrupted (Evan, M. M. S. 2007). The *ig* mutant has an extended proliferative phase during the female gametophyte generation resulting in the generation of embryo sacs with extra egg cells, central cells and polar nuclei within the central cells. The LOB domain protein encoded by *IG* is suggested to play a role in establishing polarity and possibly in regulating the expression on certain KNOTTED1-like homeobox domain transcription factors (Evan, M. M. S. 2007). When the *ig* mutant is pollinated with diploid or tetraploid pollen, the resulting seed has a diploid or triploid embryo and a range of possible endosperm karyotypes, which vary in total ploidy and in the ratio of maternal to paternal genomes. It was demonstrated that only seed with a 2m:1p genomic ratio in the endosperm can complete normal development and this is independent of the ploidy of the embryo (Lin, B. 1984). This experiment not only disproved many earlier hypotheses which attempted to explain the ploidy barrier but also demonstrated that endosperm development is strongly influenced by imprinting.

Scott et al (1998) presented the first study of interploidy crosses in *Arabidopsis*. In most *Arabidopsis* accessions, reciprocal diploid X tetraploid (2x X 4x) crosses produce viable seed and Scott et al. were able to produce a comprehensive analysis of endosperm

proliferation from interploidy crosses throughout seed development. In 2x X 4x crosses, excess paternal genomes caused accelerated endosperm mitosis, delayed cellularisation, over-proliferation of the micropylar and chalazal endosperm and high mature seed weight in comparison to balanced crosses. In contrast, in 4x X 2x crosses, excess maternal genomes caused reduced endosperm meiosis, precocious endosperm cellularization, early degradation of the micropylar and chalazal endosperm, an absence of chalazal nodes and a low mature seed weight in comparison to WT. The complementary seed phenotypes produced from interploidy crosses provide strong evidence that the maternally- and paternally- inherited genomes are not equivalent and have antagonistic affects on endosperm development. In support of the parental conflict theory these results indicate that paternally-expressed imprinting genes promote endosperm proliferation, whereas maternally-expressed imprinted genes restrict this growth. Such genes are referred to throughout this thesis as paternally-inherited endosperm-promoting genes and maternally-inherited endosperm-inhibiting genes.

1.3.3.2 *Genes subject to genomic imprinting*

Imprinting in the endosperm was conclusively confirmed by the identification of imprinted genes in *Arabidopsis* and maize. To date five imprinted genes have been identified in *Arabidopsis* and several more are known in maize. All plant imprinted genes identified to date confer parent-of-origin-specific expression during endosperm development and in support of the *parental conflict theory* most function to control endosperm development. These genes are particularly interesting because they represent potential targets for manipulation to alter seed development.

1.3.3.2.1 *Imprinted genes identified in* Arabidopsis

Imprinted genes *MEDEA* (*MEA/FIS1*) and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) are expressed in the central cell of the female gametophyte and exclusively from the maternally-inherited allele during early endosperm development (Choi, Y. et al. 2002, Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 1999, Luo, M. et al. 2000). Together with *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE/FIS3*) and *MULTICOPYSUPRESSOR OF IRA 1* (*MSI1*), *MEA* and *FIS2* are termed *FIS*-class genes (Chaudhury, A. M. et al. 1997, Grossniklaus, U. et al. 1998, Guitton, A. E. et al. 2004, Kiyosue, T. et al. 1999, Kohler, C. et al. 2003, Luo, M. et al. 1999, Ohad, N. et al. 1996). *fis*-class mutants exhibit autonomous diploid endosperm proliferation in unfertilized ovules

and when pollinated with WT pollen, embryo development arrests at around heart stage and seeds abort. This abortion is correlated with abnormal and uncontrolled endosperm proliferation indicating that *FIS*-class genes play a role in suppressing endosperm development prior to fertilization and in regulating cell proliferation during endosperm development.

MEA, FIE, FIS2 and MSI1 are Polycomb group (PcG) proteins homologous to the *Drosophila* Enhancer of Zeste E(Z), Extra Sex Combes (ESC), Suppressor of Zeste 12 SU(Z)12 and P55 respectively and are suggested to form a PCR2 type PcG complex, the MEA-FIE complex, which is similar to the animal E(Z)-ESC complex (Grossniklaus, U. et al. 1998, Guitton, A. E. et al. 2004, Kiyosue, T. et al. 1999, Köhler, C. et al. 2003, Ohad, N. et al. 1999). The animal E(Z)-ESC complex acts to repress transcription of target genes by methylating lysine 27 on histone 3 (H3K27) (Czermin, B. et al. 2002, Muller, J. et al. 2002). The maternally-expressed MEA-FIE PcG complex is therefore likely to regulate endosperm development by repressing maternally-inherited endosperm-promoting genes. In support of this model, the MEA-FIE complex has been shown to suppress the imprinted MADS-box gene *PHERES1* (*PHE1*) (Kohler, C. et al. 2003)

PHE1 is the only paternally-expressed/maternally-repressed, imprinted gene identified in *Arabidopsis* to date. In 2DAP endosperm, the paternally-expressed *PHE1* transcript is six times more abundant than the maternally-expressed transcript (Kohler, C. et al. 2005). *PHE1* expression is up-regulated in maternal *mea* mutants (Kohler, C. et al. 2003). It is therefore likely that the maternally-expressed MEA-FIE complex suppresses the maternally-inherited *PHE1* allele during endosperm development. As *PHE1* is paternally-expressed, the parental conflict theory predicts that PHE1 should act to increase endosperm proliferation. To date this is unconfirmed.

Like *MEA* and *FIS2*, *FWA* is maternally-expressed in the central cell of the female gametophyte and imprinted in the endosperm. During endosperm development *FWA* is expressed exclusively from the maternally-inherited allele (Kinoshita, T. et al. 2004). *FWA* encodes a homeodomain transcription factor and causes delayed flowering when expressed ectopically in vegetative tissue (Soppe, W. J. J. et al. 2000). A role for FWA during endosperm development remains undetermined.

The most recently identified imprinted gene is *maternally expressed PAB C-terminal* (*mPAC*) which encodes a small protein with homology to the C-terminal domain of Poly (A)-binding protein (Tiwari, S. Personal communication). *mPAC* is also maternally-expressed and paternally-silenced during early endosperm development.

1.3.3.2.2 ***Imprinted genes identified in Maize***

Several imprinted genes identified in maize are homologous to the *FIS*-class genes in *Arabidopsis* indicating that the same groups of genes may be subject to genomic imprinting throughout the plant kingdom. This is particularly interesting from a biotechnological perspective because it suggests seed development could be altered in a wide range of species by manipulating the expression of a single set of genes. Imprinted genes identified in maize are detailed below.

Similarly to the *Arabidopsis* gene *MEA*, *MEZ1* is homologous to the *Drosophila* PgC gene E (Z) (Springer, N. M. et al. 2002). *MEZ1* is expressed biallelically in embryonic tissue but imprinted during endosperm development and expressed exclusively from the maternally-inherited allele until at least 27 DAP (Haun, W. J. et al. 2007).

Two homologues of the *Arabidopsis* gene *FIE* have been identified in maize; these are named *FIE1* and *FIE2* (Springer, N. M. et al. 2002). *FIE2* is expressed in the central cell and therefore may act to suppress endosperm development before fertilization similarly to *FIE* (Danilevskaya, O. N. et al. 2003, Hermon, P. et al. 2007). *FIE2* is biallelically expressed in many tissues, including the embryo, but is imprinted during endosperm development and expressed exclusively from the maternally-inherited allele until around 5 DAP (Danilevskaya, O. N. et al. 2003, Gutierrez-Marcos, J. F. et al. 2003). *FIE1* is not expressed in the central cell but is imprinted and expressed exclusively from the maternal allele during endosperm development (Danilevskaya, O. N. et al. 2003, Gutierrez-Marcos, J. F. et al. 2006, Hermon, P. et al. 2007).

Other maternally-expressed imprinted genes in maize include *MEG1* and *NRP1*. *MEG1* is expressed exclusively from the maternally-inherited allele in endosperm from 3 to around 10 DAP (Gutierrez-Marcos, J. F. et al. 2004). Interestingly *MEG1* expression is confined to the base transfer region of the endosperm and may therefore be involved in nutrient trafficking (Gutierrez-Marcos, J. F. et al. 2004). *NRP1* encodes a putative transcription

factor and is expressed exclusively in the endosperm and from the maternally-inherited allele throughout endosperm development (Guo, M. et al. 2003).

PEG1 is the only paternally-expressed, maternally-repressed, imprinted gene identified to date in maize. *PEG1* is imprinted in the endosperm and is biallelically expressed in the embryo (Gutierrez-Marcos, J. F. et al. 2003)

1.4 *Mechanisms of genomic imprinting*

Parent-of-origin-specific DNA methylation constitutes the predominant mechanism for controlling genomic imprinting in mammals. Significant progress has been made in understanding how imprints are established and maintained in mammals. Parent-of-origin-specific DNA methylation is also fundamental for genomic imprinting in plants. The current model for how imprints are established and maintained in plants has several weaknesses, not least because it is derived from analysis of just a few imprinted genes. In this section the role of DNA methylation in controlling the expression of imprinted genes is re-assessed and revised models of genomic imprinting are proposed. This section begins by reviewing the mechanisms of genomic imprinting in mammals as this has helped in appreciating the diversity of mechanisms which could act to control genomic imprinting in plants.

1.4.1 *Mechanisms of genomic imprinting in mammals*

1.4.1.1 DNA *methylation-dependent genomic imprinting*

DNA methylation-dependent imprinting in mammals is dependent on the differential methylation of parentally-inherited genomes. Mouse embryos homozygous for a mutation in the maintenance DNA methyltransferase DNMT1 mis-express imprinted genes and fail to complete embryogenesis genes (Li, E et al. 1992, Li, E. et al. 1993). Mammalian imprinted genes are localized in clusters of three to eleven genes and expression of each cluster is primarily regulated by parent-of-origin-specific DNA methylation of an imprinting control region (ICRs) associated with each cluster (Pauler, F. M. and Barlow, D. P. 2006). Since imprints are inherited by the embryo and maintained throughout development in mammals, sex-specific imprints must be re-established in the germ line of each generation.

1.4.1.2 *Epigenetic reprogramming of DNA methylation-dependent imprints*

The mammalian epigenetic reprogramming mechanism is illustrated in **Figure 1.3.** Reprogramming begins when primordial germ cells, within the prenatal embryo, undergo a wave of genome-wide demethylation prior to meiosis. Demethylation occurs as the cells migrate into the gonad precursory tissue known as the genital ridge. During this phase, methylation in lost from both imprinted and non-imprinted loci and imprinted genes such as *H19* and *Igf2* switch from monoallelic to biallelic expression (Hajkova, P. et al. 2002, Li, J. Y. et al. 2004, Szabo, P. E. et al. 2002). The mechanism by which demethylation occurs is unknown but three possible scenarios have been suggested; demethylation may be an active process that occurs during one cell division as primordial germ cells enter the genital ridge or via a passive process initiated either prior to when the primordial germ cell enters the genital ridge or upon entry (Lees-Murdock, D. J. and Walsh, C. P. 2007). Interestingly, during this phase of demethylation, DNMT1 is still present in the nucleus of the primordial germ cells (Hajkova, P. et al. 2002). To achieve demethylation, DNMT1 must therefore either be inhibited or out-competed. Following demethylation, primordial cells in the male germ line enter a brief mitotic arrest whereas female primordial germ cells begin meiosis and then arrest at the diplotene stage of prophase 1. Female primordial germ cells remained arrested at this stage until ovulation.

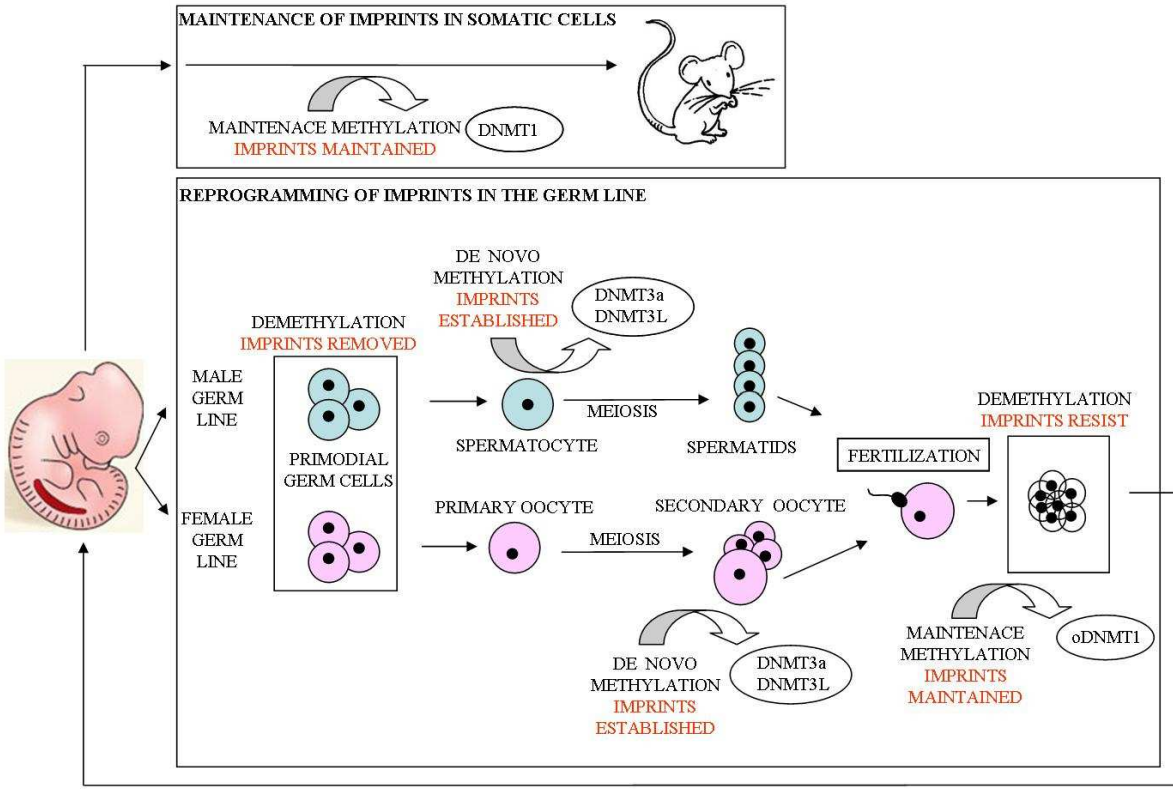


Figure 1.3 Epigenetic reprogramming of DNA methylation-dependent imprinting in mammals.

The establishment of sex-specific imprints occurs at different times in the male and female germ line. In the male germ line, imprints are established before germ cells undergo meiosis. Interestingly, analysis of the remethylation processes indicated that the paternally-inherited *H19* allele is remethylated before the maternally-inherited allele (Davis, T. L. et al. 1999, Davis, T. L. et al. 2000, Ueda, T. et al. 2000). This suggests that either the paternally-inherited allele has retained a signal to be methylated first or the maternally-inherited allele has retained a signal to initially resist methylation. Either way this finding indicates that one or both parentally-inherited alleles retain some kind of epigenetic memory that is independent of DNA methylation.

In the female germ line, the remethylation of imprinted loci occurs when the germ cell ceases meiotic arrest and enters the postnatal oocyte growth phase. A number of imprinted genes in the female germ line are also remethylated in a parent-of-origin-specific manner. For example the maternally-inherited alleles of *Snrpn*, *Zac1* and *Peg1* are remethylated before the paternally-inherited alleles (Hiura, H. et al. 2006, Lucifero, D. et al. 2004). Again this suggests that imprinted alleles retain some epigenetic memory that is

independent of methylation. It is suggested that these alleles may be marked by parent-of-origin-specific chromatin modifications during germ cell development and these modifications are maintained when methylation is erased in primordial germ cells (Lucifero, D. et al. 2004).

Imprints are established by the *de novo* methyltransferase activity of DNMT3 and are alsodependent on DNMT3L, a protein sharing homology with DNA methyltransferases but lacking enzymatic activity. Homozygous *dnmt3a* mutant mice are normal at birth but die after four weeks (Okano, M. et al. 1999). Offspring of maternal *dnmt3a* mutants die *in utero* and lack methylation at, and monoallelic expression of, imprinted loci (Kaneda, M. et al. 2004). Male *dnmt3a* mutants are infertile and fail to methylate imprinted loci in the germ line (Kaneda, M. et al. 2004). Similarly offspring of maternal *dnmt3L* mutants die *in utero* and lack methylation and allele-specific expression of imprinted loci, whereas male *dnmt3L* mutants are sterile (Bourc'his, D. et al. 2001, Hata, K. et al. 2002).

During different stages of gametogenesis, *DNMT1* undergoes germ line-specific alternative splicing. In the male germ line, *DNMT1* transcript is detectable at high levels until the pachytene stage of meiosis 1. Subsequently alternative splicing gives rise to *DNMT1p* mRNA which is not translated (Mertineit, C. et al. 1998). In the female germ line, towards the end of the oocyte growth phase, alternative splicing gives rise to *DNMT1o* mRNA. DNMT1o is excluded from the nucleus and restricted to the cytoplasm (Mertineit, C. et al. 1998). DNMT1o remains cytoplasmic in the preimplantation embryo, except for a brief period at the 8-cell stage, and is required to maintain the methylation at imprinted loci during early zygotic development (Howell, C. Y. et al. 2001). In female *dnmto* homozygous mutants imprints are normally established in oocytes but are lost in zygotes; this results in the loss of monoallelic expression.

Before implantation, the embryo undergoes a second wave of demethylation but critically the imprinted loci are immune to this. This second phase of demethylation is reviewed by Riek et al. (2001). After implantation imprints are maintained during somatic development by DNMT1 (Li, E. et al. 1993). The DNA methylation-dependent imprinting system in mammals is therefore dependent on maintenance and *de novo* DNA methyltransferase activity.

1.4.1.3 *Methylation-independent genomic imprinting in mammal*

Imprinting at some loci in mammals is independent of DNA methylation and instead dependent on histone modifications. For example, a collection of placenta specific, maternally expressed imprinted genes in the IC2 cluster retain maternal specific expression in placenta of homozygous *dnmt1* mutant embryos (Lewis, A. et al. 2004). These loci exhibit allelic differences in histone methylation and acetylation and disruption of these correlated with loss of imprinting (Lewis, A. et al. 2004).

1.4.2 *Mechanisms of genomic imprinting in plants*

In this section, evidence for the role of DNA methylation in plant genomic imprinting is reviewed and the potential role of different plant DNA methyltransferases in imprinting is considered. Subsequently current model of the imprinting mechanism in plants is evaluated and two novel models for DNA-dependent genomic imprinting are proposed.

1.4.2.1 *Evidence of DNA methylation-dependent genomic imprinting in plants*

A fundamental study implicating the involvement of DNA methylation in plant genomic imprinting was carried out by Adams et al (2000). Adams et al. performed reciprocal crosses between WT plants and plants transformed with an antisense (as) sequence of the major plant maintenance DNA methyltransferase gene *DNA METHYLTRANSFERASE 1* (*MET1)*. *MET1*as plants have genome-wide hypomethylation predominately at CG dinucleotides (Ronemus, M. J. et al. 1996). Adams et al. observed that [*MET1*as X WT] seed displays a paternal excess phenotype similar to that of [2x X 4x] seed; [*MET1*as X WT] seed has a high mature seed weight and increased endosperm proliferation compared to WT seed. In contrast [WT X *MET1*as] seed displays a maternal excess phenotype similar to that of [4x X 2x] seed; [WT X *MET1*as] seed has a low mature seed weight and decreased endosperm proliferation compared to WT seed. Maternal inheritance of hypomethylated genomes therefore causes a similar phenotypic effect to inheritance of excess paternal genomes and conversely paternal-inheritance of hypomethylated genomes causes a similar effect to inheritance of excess maternal genomes. This indicates that parent-of-origin-specific expression is dependent on the inheritance of parent-of-origin specific DNA methylation patterns. It is concluded that DNA methylation acts to silence maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes.

Further evidence that methylation of the paternally-inherited genome silences endosperm-inhibiting genes comes from the observation that hypomethylated pollen rescues seed abortion of certain maternal *fis*-class mutants. As discussed above, the maternal inheritance of a *fis*-class mutant results in endosperm over-proliferation and seed abortion (1**.3.3.2.1**). This phenotype is similar to that of paternal excess suggesting that seed abortion of *fis*-class mutants is caused by the released silencing of maternally-inherited endosperm-promoting genes (Spielman, M. et al. 2001). Fertilization of *mea* mutants with pollen from the hypomethylated *ddm* mutant rescues seed abortion (Vielle-Calzada, J. P. et al. 1999). Similarly, fertilization of *fie* mutants with *MET1*as pollen also rescues abortion (Vinkenoog, R. et al. 2000). This indicates that hypomethylated paternally-inherited genomes inhibiting endosperm proliferation and can counteract the effects caused by maternal *fis*-class mutants and restore a viable level of endosperm growth.

In addition to the evidence presented above, the requirement for DNA methylation in controlling locus-specific imprinting has been demonstrated. This is discussed in **1.4.2.4**.

1.4.2.2 *Plant DNA methyltransferases*

In plants, cytosine in any sequence context can be methylated but methylation at CpG and CpNpG sequences is most common (Chan, S. W. L. et al. 2005, Goll, M. G. and Bester, T. H. 2005). Plants have the full complement of the DNA methyltransferases found in mammals and a unique plant family. Plant DNA methyltransferases can be divided into three families; the *DNA METHYLTRANSFERASE1* (*MET1*) gene family, the *DOMAINS REARRANGED METHYLTRANSFERASE* (*DRM*) gene family and the *CHROMOMETHYLTRANSFERASE* (*CMT*) gene family. *MET1* family genes are homologous to the mammalian maintenance DNA methyltransferase *DNMT1*, *DRM* family genes are homologous to the mammalian *de novo* DNA methyltransferases *DNMT3a* and *DNMT3b* and *CMT* family genes are unique to plants as they have a chromodomain between motifs II and IV of the methyltransferase catalytic domain (Henikoff, S. and Comai, L. 1998). All the DNA methyltransferases identified in *Arabidopsis* are detailed below.

The *MET1* family comprises *MET1*, *MET2a*, *MET2b* and *MET3* (Finnegan, E. J. and Dennis, E. S. 1993, Genger, R. K et al. 1999). Antisense suppression of *MET1* causes a 90 % reduction in CG methylation (Ronemus, M. J. et al. 1996), indicating that *MET1*

encodes the major plant maintenance DNA methyltransferase of GC methylation and is therefore functionally homologous to *DNMT1*. MET1 is critical for regulating plant development; its suppression causes a variety of developmental and phenotypic abnormalities including altered flowering time and stature, homeotic transformation of floral organs and reduced fertility (Finnegan, E J. et al. 2000, Finnegan, E. J. et al. 1996, Ronemus, M. J. et al. 1996).

MET2a, *MET2b* and *MET3* were identified by Southern hybridisation and database scanning using probes homologous to *MET1* (Genger, R. K et al. 1999). The *MET1* family members are highly similar and are derived from the duplication of a *MET1*-like ancestral (Finnegan, E. J. and K 2000, Genger, R. K et al. 1999). MET2a and MET2b are the most similar and share more than 90 % amino acid sequence homology in both their amino terminal and methyltransferase domains (Finnegan, E. J. and K 2000). Transcripts from either *MET2a* and/or *MET2b* are detectable at a low level in vegetative and floral tissue of the accession C24 (Genger, R. K et al. 1999). In contrast *MET3* is suggested to encode a truncated protein in the *Arabidopsis* accession Columbia (Col-0) due to the presence of a stop codon within the coding region (Genger, R. K et al. 1999). However the absence of this stop codon in the *Arabidopsis* accession Landsberg-erecta (La-*er*) indicates that *MET3* may encode a functional protein in other accessions (Finnegan, E. J. and Kovac, K. A. 2000). No expression studies have been reported for *MET3*. The function of *MET2a*, *MET2b* and *MET3* is unknown, but all three genes are annotated as putative maintenance DNA methyltransferases.

The *CMT* family consists of *CMT1*, *CMT2*, and *CMT3* but only *CMT3* encodes a functional DNA methyltransferase (Finnegan, E. J. and Kovac, K. A. 2000, Genger, R. K et al. 1999, McCallum, C. M. et al. 2000). CMT3 is required for the maintenance of methylation at non-GC loci and *cmt3* mutants have reduced cytosine methylation at CNG and asymmetric sequences of transgenes and endogenous sequences (Bartee, L. et al. 2001, Lindroth, A. M. et al. 2001).

The *DRM* family includes *DRM1* and *DRM2* (Cao, X. et al. 2000). Similarly to the mammalian DNMT3s, DRMs have *de novo* methyltransferase activity. The *drm1drm2* double mutant is defective in *de novo* methylation of transgenes at CG, CNG and asymmetric sequences (Cao, X. and Jacobsen, S. E. 2002b). DRMs act downstream of

small RNAs to achieve RNA-directed DNA methylation at CNG and asymmetric sequences and acts redundantly to CMT3 to maintain methylation at these sequences (Cao, X. et al. 2003, Cao, X. and Jacobsen, S. E. 2002a). Neither *drm1drm2* double mutant nor *cmt3* mutants display developmental abnormalities although the *drm1drm2cmt3* triple mutant displays pleiotrophic effects on plant development (Cao, X. and Jacobsen, S. E. 2002a).

1.4.2.3 *The role of DNA methyltransferases in genomic imprinting: A putative role for MET2a, MET2b and MET3*

CMT and *DRM* family genes apparently play a role in plant genomic imprinting. No parent-of-origin-specific affects on seed size are observed from reciprocal crosses between *cmt3* or *drm1drm2* mutants and WT plants (Xiao, W. et al. 2006a) suggesting that these genes are not required to maintain the silencing of endosperm-promoting or –inhibiting genes. Additionally imprinting of the *FWA* locus is not disrupted in *cmt3* and *drm1drm2* mutants (Kinoshita, T. et al. 2004) and there is no evidence that CM3 or DRM family genes play a role in controlling the expression of this locus.

As suggested above (**1.4.2.1**), the parent-of-origin-specific affects on seed development caused by *MET1* suppression have substantiated a role for *MET1* in controlling imprinting gene expression (Adams, S. et al. 2000). However, because the work depended on the analysis of lines in which *MET1* is suppressed, and hypomethylation is induced, throughout the entire lifecycle of the plant, little is know about when MET1 is required for imprinting. Additionally, no evaluation of a potential role of MET2a, MET2b and MET3 in genomic imprinting has been reported. As MET1 family members have high amino acid sequence homology it is likely that these protein have similar functionality and it is possible therefore that MET2a, MET2b and MET3 also play a role in controlling imprinted gene expression. Below it is argued that a putative role for MET2a, MET2b and MET3 in genomic imprinting may be masked in the study of *MET1*as lines.

Much of the work implicating MET1 in genomic imprinting is based on the study of *MET1*as lines. Antisense expression of a gene can cause off-target suppression of genes with high DNA sequence homology to the target; consequently gene families are particularly vulnerable to off target effects of antisense suppression (Tada, Y. et al. 2003). The affect of sequence homology on antisense suppression has been investigated using the

chalcone synthase gene family in *Gerbera hybrida* (Elomaa, P. et al. 1996). Two chalcone synthase family genes, *GCHS1* and *GCHS3*, share 78% homology at the nucleic acid level and both genes share 73% homology with a third gene family member, *GCHS2*. Antisense expression of *GCHS1* causes equal suppression of *GCHS1* and *GCHS3*, and suppression of *GCHS2* in a number of transformants. Similarly, antisense expression of *GCHS2* causes suppression of *GCHS1* and *GCHS3* in a number of transformants. Antisense expression of a gene can therefore cause off-target suppression of genes sharing as little as 73 % nuclei acid sequence homology with the target. *MET1* shares 78%, 78% and 70% nucleic acid sequence homology with *MET2a*, *MET2b* and *MET3* respectively. It is therefore possible *MET1*as expression causes suppression all four *MET1* family genes. Putative *MET2a*, *MET2b* and *MET3* suppression could account for some of the parent-of-origin-specific effects reported in *MET1* as lines.

MET2a, MET2b and MET3 could be involved in genomic imprinting in a number of possible ways. MET2a, MET2b and/or MET3 could act redundantly to MET1 in silencing maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes. Alternatively, they could have tissue-specific roles in maintaining the silencing of imprinted genes. In mammals, *DNMT1* undergoes alternative splicing to produce tissue-specific DNMT1 transcripts (See above section **1.4.1.2**). It is possible that plant genomic imprinting also requires tissue-and transcript-specific maintenance DNA methyltransferase activity. This could be achieved by the tissue-specific expression of *MET1* family genes rather than the alternative splicing of one transcript. The spermatocyte-specific *DNMT1p* transcript is not translated but may still have a function in mammals. If *MET3* encodes a non-functional protein it is possible that the transcript still plays some role in imprinting; for example it may act to suppress expression of other *MET1* family genes. Finally, it is possible that *MET2a*, *MET2b* and/or *MET3* function in a locus-specific manner to maintain the silencing of specific imprinted genes independently of *MET1*.

1.4.2.4 *Current models for the control of genomic imprinting in plants*

The current model for the control of genomic imprinting in plants incorporates both DNA methylation-dependent and histone modification-dependent mechanisms. These are considered separately.

1.4.2.4.1 *DNA methylation-dependent genomic imprinting*

The current model for the DNA methylation-dependent mechanism of genomic imprinting in plants is based on the mechanism resolved for *FWA* and *FIS2*. The default state of *FWA* and *FIS2* is methylated and silent. Ectopic vegetative expression of *FWA* is induced in hypomethylated *ddm1* mutants and is correlated with loss of methylation at two tandem repeats 5' to the *FWA* transcription start (Soppe, W. J. J. et al. 2000). This indicates that vegetative silencing of *FWA* is dependent on DNA methylation. In agreement with this, targeted remethylation of the *FWA* tandem repeats is sufficient to silence ectopic *FWA* expression (Kinoshita, Y. et al. 2007). Vegetative silencing of *FIS2* is also correlated with methylation of a 200 bp region 5' to the *FIS2* transcriptional start (Jullien, P. E. et al. 2006b). Methylation of this regions in not necessary for vegetative *FIS2* silencing because ectopic expression is not induced in hypomethylated mutants (Jullien, P. E. et al. 2006b). However, methylation of this region is likely to be necessary for *FIS2* imprinting, as explained below.

Silencing of *FWA* and *FIS2* is released in the central cell of the female gametophyte and both genes are imprinted and exclusively maternally-expressed allele during endosperm development (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). Released silencing of *FWA* and *FIS2* is dependent on the central cell-specific DNA glycosylase *DEMETER* (*DME*) (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). *dme* mutants have reduced *FWA* and *FIS2* expression before and after fertilization (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). DME excises 5'methylcytosine *in vitro* and when expressed in *E. coli* (Gehring, M. et al. 2006) and is therefore likely that DME releases silencing of *FWA* and *FIS2* by excising methylated cytosines from the differentially methylated regions (DRMs) associated with these loci.

Paternal-silencing of *FWA* and *FIS2* in the endosperm is apparently maintained by maintenance DNA methyltransferase activity, which is also suggested to maintain methylation at the DMRs associated with theses loci throughout vegetative development and during the male gametophyte generation (Jullien, P. E. et al. 2006b). In agreement with this, paternal-silencing of *FWA* and *FIS2* is lost when these alleles are inherited from hypomethylated *met1* mutants (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004).

The DNA methylation-dependent mechanism of the current model of genomic imprinting is summarised in **Figure 1.4**. A role for *MET2a*, *MET2b* and *MET3* in genomic imprinting has been hypothesised (**1.4.2.3**) and therefore a putative role for these genes has been included in the model where *MET1* alone was suggested to act.

A similar DNA methylation-dependent mechanism may control imprinting of the maternally-expressed maize gene *FIE1*. Silencing of *FIE1* in vegetative tissue and during endosperm development is correlated with methylation of DMRs associated with this locus (Hermon, P. et al. 2007). These DMRs are demethylated in the central cell but not in the sperm cells (Gutierrez-Marcos, J. F. et al. 2006). Parent-of-origin-specific methylation is maintained during endosperm development and is correlated with the mono-allelic expression of *FIE1* (Gutierrez-Marcos, J. F. et al. 2006, Hermon, P. et al. 2007). However, in contrast to *FWA* and *FIS2*, *FIE1* is not expressed in the central cell and expression from the maternally-inherited allele first detected 24-32 hours after fertilization (Hermon, P. et al. 2007). This indicates that demethylation of DMRs at the *FIE1* locus may be necessary but not sufficient to release silencing.

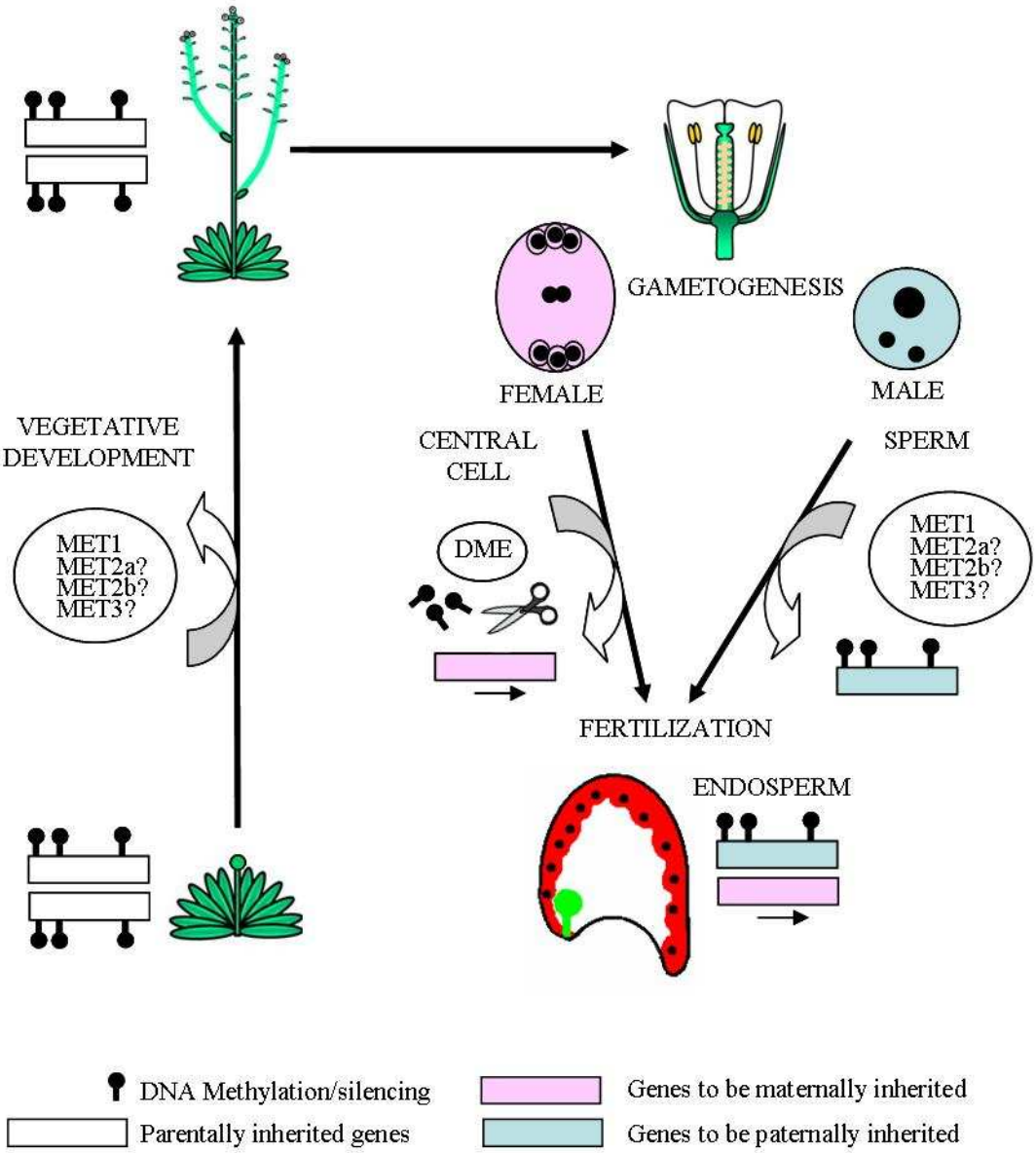


Figure 1.4 The current DNA methylation-dependent model of genomic imprinting in plants. Maintenance of methylation at imprinted loci is maintained throughout vegetative development by maintenance DNA methyltransferases. Methylation and silencing is removed from maternal alleles by DME in the central cell. In contrast, methylation and silencing is maintained at paternal alleles by maintenance DNA methyltransferases. Consequently gametes are differentially methylation and imprinting occurs in during endosperm development. This model is adapted from (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004).

1.4.2.4.2 *Histone modification-dependent genomic imprinting*

Like *FWA* and *FIS2*, expression of *MEA* in the central cell, and from the maternally-inherited allele during endosperm development, is dependent on DME and associated with loss of methylation at DMRs associated with the *MEA* locus (Choi, Y. et al. 2002, Gehring,

M. et al. 2006). However, paternal inheritance of *MEA* from a hypomethylated background does not disrupt *MEA* imprinting (Gehring, M. et al. 2006, Jullien, P. E. et al. 2006b) indicating that silencing of the paternally-inherited allele is not dependent on DNA methylation. Interestingly, ectopic expression of the paternal *MEA* allele is detected in seed derived from *mea* or *fie* mutants (Gehring, M. et al. 2006). Moreover, the paternal *MEA* allele is enriched with histone H3 lysine 27 (H3K27) methylation compared to the maternal allele (Gehring, M. et al. 2006). These findings suggest that the paternal *MEA* allele is silenced by H3K27 methylation which is maintained by the maternally-expressed MEA-FIE complex.

Imprinting of *PHE1* is also dependent on allele-specific histone modifications. MEA and FIE bind directly to the *PHE1* promoter and *PHE1* silencing is relaxed in *mea* mutants (Kohler, C. et al. 2003, Kohler, C. et al. 2005). These result indicate that silencing of the maternally PHE1 allele is also dependent on the MEA-FIE complex. It is possible that this complex also regulates the expression of other imprinted genes.

The activity of DME and the MEA-FIE complex is central to the DNA methylation-dependent and histone methylation-dependent mechanisms proposed in the current model of genomic imprinting. This model therefore emphasises that genomic imprinting is maternally controlled. To reflect this, the control of plant genomic imprinting has been referred to as the Oedipus complex of flowering plants (Autran, D. et al. 2005).

1.4.2.5 *Problems with the current model for the control of genomic imprinting*

Two potential weaknesses in the current model for the control of genomic imprinting in plants are apparent. Firstly, the model is largely dependent on genes that were identified from very similar mutant screens. All components of the MEA-FIE PgG complex were initially identified because of their *fis* mutant phenotype i.e. over-proliferated endosperm and seed lethality. These similar mutant phenotypes indicated that these genes may act in the same pathway; as was identified. The same research groups which identified the role of DME in controlling the imprinting of *fis*-class genes also demonstrated that *FWA* imprinting is controlled by a similar mechanism (Choi, Y. et al. 2002, Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). This concentrated effort has accelerated our understanding of plant genomic imprinting but it must be questioned whether a general mechanism or an exceptional pathway of genomic imprinting has been uncovered. The

identification of more imprinted genes in plants will help resolve this problem. At present it may be wise to be sceptical of the validity of this model.

Secondly, the current model of genomic imprinting emphasises the maternal control of imprinting; this may represent only half the picture and it is proposed here that a paternal control mechanism also exists - an Electra complex to complement the Oedipus complex. The complementary seed phenotypes produced from reciprocal crosses between *MET1*as and WT plants indicate that DNA methylation silences maternally-inherited endosperm-inhibiting genes as well as paternally-inherited endosperm-promoting genes. The current model of genomic imprinting does not address how plants achieve the silencing of maternally-inherited endosperm-inhibiting genes and the expression of paternally-inherited endosperm-promoting genes. The parental conflict theory proposes that the paternally-inherited genes evolved to promote endosperm proliferation as a consequence of a fitness advantage to the pollen parent. It is therefore logical that a paternally-controlled mechanism would have evolved to ensure the expression of these. It is also possible that a paternally-controlled mechanism exists to suppress maternally-inherited endosperm-inhibiting genes.

It should also be noted that the requirement of DNA methyltransferase activity predicted by the model has yet to be validated as only lines in which *MET1* is suppressed throughout development have been available to date.

1.4.2.6 *Alternative mechanisms for DNA methylation-dependent genomic imprinting*

A variety of DNA methylation-dependent mechanisms could achieve genomic imprinting and different mechanisms could act to control expression at different imprinted loci. This section considers a few possible mechanisms and proposes two possible models which have different tissue-specific requirements for maintenance DNA methyltransferase activity.

Parent-of-origin-specific effects resulting from the inheritance of hypomethylated genomes indicates either that genomes become differential methylation prior to fertilization or that DNA methylation is essential for the establishment of a second epigenetic modification which determines parent-of-origin-specific expression after fertilization.

The mechanism, by which parental genomes could be differentially methylated, may depend on whether imprinted loci are methylated or unmethylated as default i.e. throughout vegetative development. If imprinted loci are unmethylated as default, imprints must be established by sex-specific *de novo* methylation. This is how the two parental genomes are differentially methylated in mammals (See section **1.4.1.1** and **1.4.1.2**). However, analysis of *de novo* methyltransferase mutants has revealed no evidence of altered imprinting, indicating that *de novo* methyltransferases activity plays no detectable role in controlling imprinting in plants (see **1.4.2.3**). On the other hand, there is some evidence that *de novo* methylation may be involved in *FIE2* imprinting in maize (Gutierrez-Marcos, J. F. et al. 2006). *FIE2* imprinting is correlated with the parent-of-origin-specific methylation of a DRM associated with this locus. The DMR of the paternal *FIE2* allele is unmethylated in sperm cells but methylated in endosperm suggesting it must receive *de novo* methylation. However, it is recognised that the imprinting of *FIE2* may be independent of DNA methylation and dependent on a different epigenetic signal (Gutierrez-Marcos, J. F. et al. 2006). In light of the evidence, the establishment of DNA methylation-dependent imprints by *de novo* methylation in plants appears unlikely.

An alternative possibility assumes that imprinted loci are methylated as default, and that imprinted loci are subject to sex specific demethylation. This is the mechanism by which imprinting of *FIS2* and *FWA* is achieved (see **1.4.2.4**). If the default state of imprinted genes is methylated it is conceivable that maintenance DNA methyltransferase activity will be required to maintain methylation at the imprinted loci throughout vegetative development and until imprints are set. Additionally, maintenance DNA methyltransferase activity may be required to maintain methylation at the silenced allele throughout the gametophyte generation and until imprinting ceases. If the imprinting of both maternally-expressed and paternally-expressed imprinted genes is controlled by this mechanism maintenance DNA methyltransferase activity may be required throughout the entire life cycle of the plant.

A mechanism to demethylate maternal alleles has been exemplified by the activity DME and other maternal mechanisms may also exist. However, no mechanism has been identified to demethylate paternal alleles. *DME* is expressed the central cell-specifically which rules out the possibility that DME could act paternally. There is some evidence that the male gametophyte undergoes a wave of demethylation prior to gametogenesis. In

tobacco, the generative cells is reportedly has eighty percent less methylated than the vegetative cell (Oakeley, E. J. et al. 1997) and in *Lilium longiflorum* the generative cell is reportedly hypomethylated in comparison to other cells within the anther (Janousek, B et al. 2000). Hypomethylation of the generative cell could provide a mechanism to demethylation of paternal alleles.

If genomic imprinting depends on the differential methylation of parental genomes, these must be differentially methylated prior to fertilization. As most plants are hermaphrodites, this must occur at some point between the divergence of the male and female sexual organs and the fusion of male and female gametes. This period can be divided into two developmental windows; 1) the sporophytic development of anthers and carpels, from the divergence of floral organ primordia to post-meiotic microspore and megaspore production and 2) the gametophyte generation, from sporogenesis to gamete formation.

In flowering plants, evidence to date suggests that imprinting is restricted to the endosperm. If imprints are set prior to gamete formation they are likely to be inherited by the gamete destined to form the embryo as well as that destined to form the endosperm. To achieve imprinting only in the endosperm, imprints must be set after gamete formation and only in the gamete destined for the endosperm. Alternatively, if imprint are set prior to gamete formation, imprints may need to be removed from the gamete destined for the embryo, prior to or after fertilization.

The central cell-specific activity of DME, indicates that demethylation of maternal alleles can be restricted to the central cell. It is more difficult to conceive how demethylation of paternal alleles could be restricted to the sperm cell destined for the endosperm because many species appear to have isomorphic sperm. However, sperm dimorphism has been reported in some species e.g. *Torenia fournieri* (Chen, S. H. et al. 2006) and tobacco (Tian, H. Q. et al. 2001); sperm-specific demethylation may therefore be possible. In mammals imprints are set prior to or during meiosis (**1.4.1.2**), it is therefore possible that some imprints are prior to or during sporogenesis in plants.

As suggested above, it is also possible that imprinting at some loci is dependent on the differential modification of alleles by an epigenetic modification other than DNA methylation, but which is dependent DNA methylation for its establishment. Alleles could

be sex-specifically 'marked' prior to or during meiosis to ensure that they are silenced in the endosperm. This mechanism would guarantee imprints are faithfully transmitted to gametes irrespective of any global changes in methylation that may occur during the gametophyte generation. These 'marks' could signal the methylation and silencing of alleles after fertilization. In mammals, some genes are remethylated in a parent-of-origin-specific manner which indicates that alleles retain some kind of epigenetic memory of their methylation status (**1.4.1.2**); a similar phenomenon could therefore also occur in plants. This mechanism could explain how imprinting of the maize gene FIS2 is achieved.

Based on this discussion two models to account for the possible mechanisms by which imprinting could be achieved are presented here. In model 1 (**Figure 1.5**) the default state of imprinted genes is methylation and sex-specific demethylation causes the differential methylation of male and female genomes. This model requires maintenance DNA-methyltransferase activity throughout development. Similarly, in model 2 (**Figure 1.6**), the default state of imprinted genes is methylation and sex-specific demethylation causes the differential methylation of male and female genomes. However, alleles are also sex-specifically 'marked' by an epigenetic modification that ensures they are silenced in the endosperm. This mechanism requires maintenance DNA methyltransferase activity throughout sporophyte development and until meiosis to ensure that the epigenetic 'marks' can be established, but is independent of maintenance DNA methyltransferase activity during the gametophyte generation. It should be noted that models 1 and 2 are not mutually exclusive nor do they attempt to explain how DNA methylation-dependent imprinting at all loci is controlled. Instead, they extend and challenge the current model of genomic imprinting and emphasis that MET1 may not be required throughout development to maintain the imprinting of all loci.

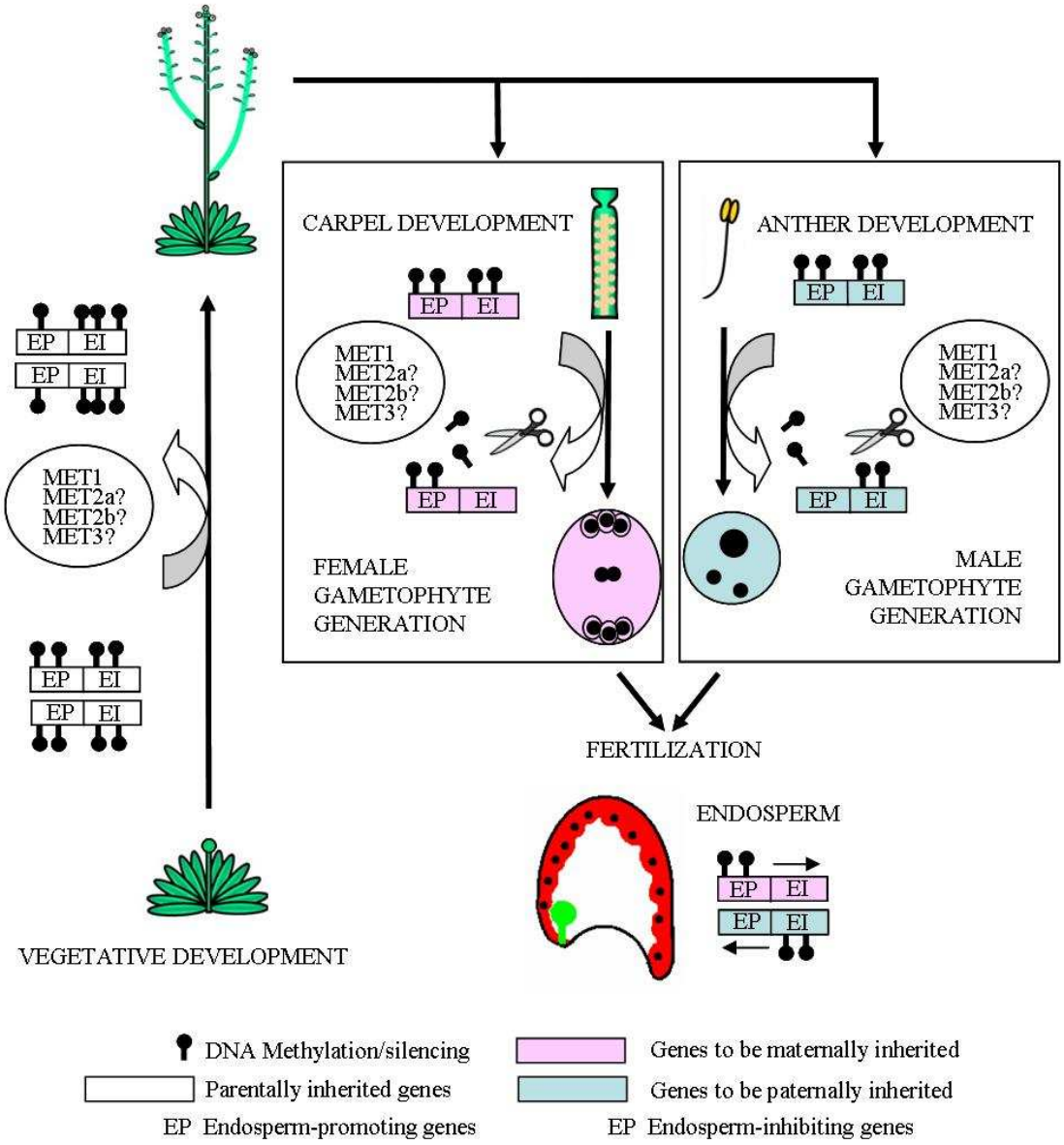


Figure 1.5 Model 1: a putative mechanism for DNA methylation-dependent genomic imprinting: The maintenance DNA methyltransferase activity, by MET1, MET2a, MET2b and/or MET3, maintains the default methylated state of imprinted genes throughout vegetative development. During carpel development, or the female gametophyte generation, endosperm-inhibiting genes are demethylated and methylation of endosperm-promoting genes is maintained by maintenance DNA methyltransferase activity. Conversely, during anther development, or the male gametophyte generation, endosperm-promoting genes are demethylated and methylation of endosperm-inhibiting genes is maintained by maintenance DNA methyltransferase activity. Consequently endosperm-inhibiting and promoting genes are sex-specifically methylated in gametes and expressed in a parent-of-origin-specific manner in the endosperm.

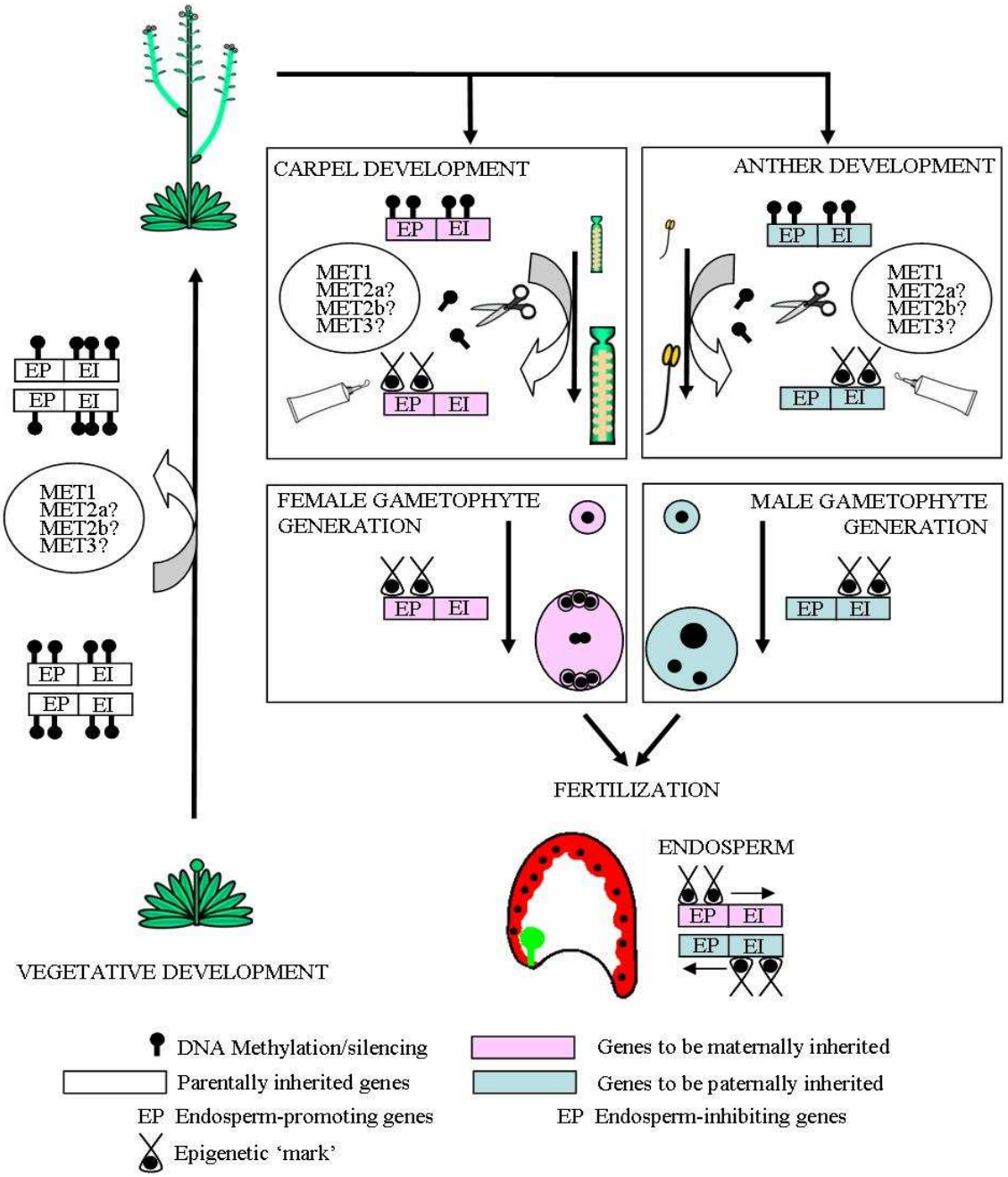


Figure 1.6 Model 2: A putative mechanism for DNA methylation-dependent genomic imprinting. Maintenance DNA methyltransferase activity, by MET1, MET2a, MET2b and/or MET3, maintains the default methylated state of imprinted genes throughout vegetative development. During carpel development, prior or during meiosis, endosperm-inhibiting genes are demethylated and endosperm-promoting genes are 'marked' by an epigenetic modification. Conversely, during anther development, prior to or during meiosis, endosperm-promoting genes are demethylated and endosperm-inhibiting genes are 'marked' by an epigenetic modification. These epigenetic 'marks' are maintained throughout the gametophyte generation independently of maintenance DNA methyltransferase activity and ensure allele are silenced in a parent-of-origin-specific manner during endosperm development.

1.4.2.7 *Testing the requirement of maintenance DNA methyltransferases for genomic imprinting*

As exemplified by models 1 and 2, different mechanisms of genomic imprinting could have different tissue-specific requirements for maintenance DNA methyltransferase activity. A greater understanding of DNA methylation-dependent imprinting could therefore be gained by determining when maintenance DNA methyltransferase activity is required for imprinting; this could be investigated by suppressing maintenance DNA methyltransferase activity tissue-specifically and testing the affects on imprinting. Moreover, models 1 and 2 make testable predictions about the tissue-specific requirement of maintenance DNA methyltransferase activity for imprinting and this approach could also be used to test the validity of these models. The effects of tissue-specific suppression of maintenance DNA methyltransferases on imprinting predicted by models 1 and 2 is described below.

Model 1 predicts that maintenance DNA methyltransferase activity is required throughout development. **Figure 1.7** illustrates the predicted effects of maintenance DNA methyltransferase suppression on the imprinting of endosperm-promoting genes. Suppression of maintenance DNA methyltransferases during carpel development, or in the female gametophyte, is predicted to result in the biallelic expression of endosperm-promoting genes in the endosperm of seed derived from these plants (**Figure 1.7b**). This seed is therefore predicted to be epigenetically similar to [*MET1*as X WT] seed and have increased seed weight. In contrast, suppression of maintenance DNA methyltransferases during anther development, or in the male gametophyte, is predicted to have no effect on the imprinting of these genes (**Figure 1.7c**). Suppression of maintenance DNA methyltransferase activity is predicted to have the reciprocal effect on the expression of endosperm-inhibiting genes. Suppression during anther development, or in the male gametophyte, is predicted to result in the biallelic expression of endosperm-inhibiting genes and in seed derived from these plants is predicted to be epigenetically similar to [WT X *MET1*as] seed and have decreased seed weight. In contrast, suppression during carpel development, or in the female gametophyte, is predicted to have no effect on the imprinting of these genes.

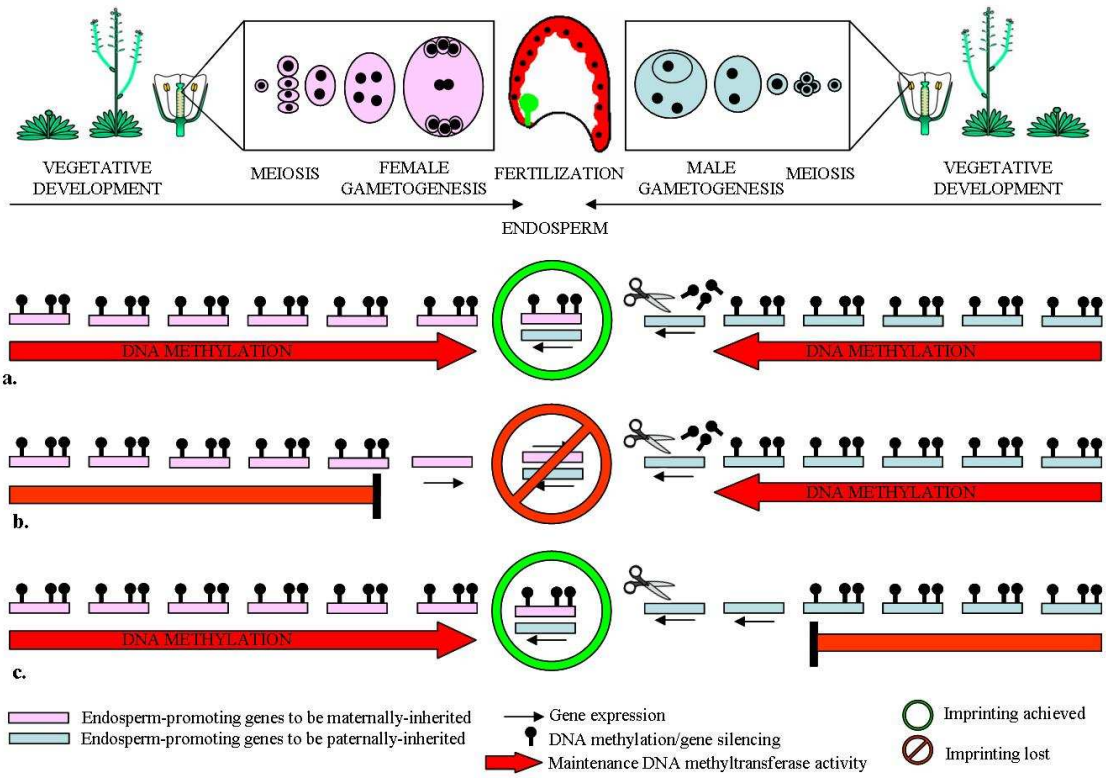


Figure 1.7. The effects of tissue-specific hypomethylation of the imprinting of genes controlled by the mechanism proposed in Model 1. a. The imprinting mechanism of endosperm-promoting genes. **b.** Maintenance DNA methyltransferase suppression during carpel development, or the female gametophyte generation, results in the biallelic expression of endosperm-promoting genes. **c.** Maintenance DNA methyltransferase suppression during anther development, or the male gametophyte generation, has no effect on the imprinting of endosperm-promoting genes.

Model 2 predicts that imprinting is dependent on maintenance DNA methyltransferase activity only until meiosis. **Figure 1.8** illustrates the predicted effects of maintenance DNA methyltransferase suppression on the imprinting of endosperm-inhibiting genes. Suppression during anther development is predicted to cause biallelic expression of endosperm-inhibiting genes in the endosperm of seed derived from these plants (**Figure 1.8b**). Consequently, this seed will have decreased seed weight. In contrast, suppression only in the male gametophyte generation is predicted to have no effect on the imprinting of these genes (**Figure 1.8c**). Conversely, suppression during carpel development is predicted to cause biallelic expression of endosperm-promoting genes and cause increased seed weight. In contrast, suppression during the female gametophyte generation only is predicted to have no effect on the imprinting of these genes.

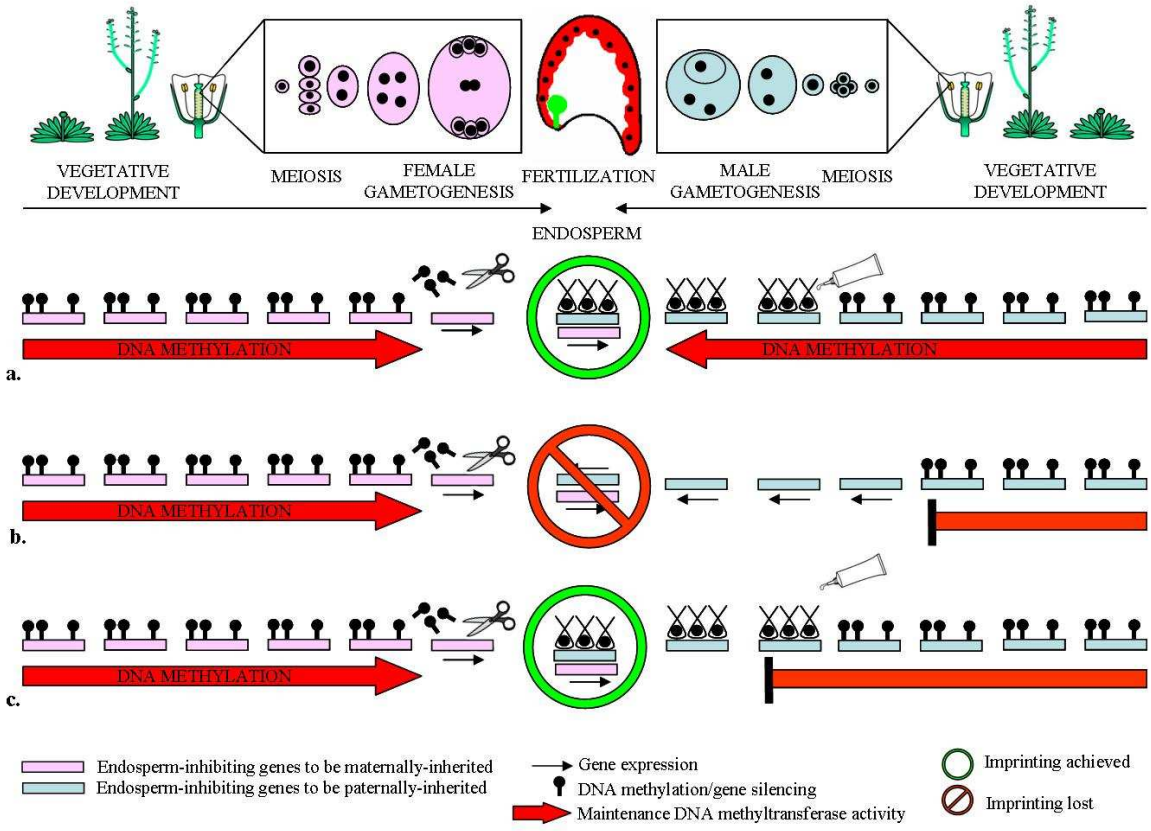


Figure 1.8 The effects of tissue-specific hypomethylation of the imprinting of genes controlled by the mechanism proposed in Model 2. a. The imprinting mechanism of endosperm-inhibiting genes **b.** Suppression of maintenance DNA methyltransferases during anther development causes biallelic expression of endosperm-inhibiting gene. **c.** Suppression of maintenance DNA methyltransferases during male gametophyte generation has no effect on the imprinting of endosperm-promoting genes.

1.5 *Altering seed size in agriculturally important species*

The capability to alter seed size has important potential agricultural applications beyond increasing yield. Seed size influences the ability of seedlings to establish and grow under different environmental stresses. Seedlings from larger seeds have access to more storage reserves and therefore have greater tolerance to nutrient deprivation compared to those from small seed (Krannitz, P. G. et al. 1991). Additionally, they have higher resilience to seed predators than seedlings from small seeds (Mack, A. L. 1998). However, seedlings from small seeds have a greater tolerance to short term drought that those from large seeds because their reduced surface area restricts water loss by transpiration (Hendrix, S. D. et al. 1991).

Adams et al (2000) demonstrated that inheritance of hypomethylated genomes has reciprocal parent-of-origin-specific effects on seed size. As implied above (**1.4.2.7**), it may therefore be possible to alter seed size in agriculturally important species by manipulating parent-of-origin-specific methylation. However transferring this concept into an agricultural commodity poses many challenges. Some of these are discussed below.

At present the ability to alter seed size in the progeny of *MET1*as plants is dependent on manual crossing with WT plants. This poses two problems; 1) crossing plants manually is impractical on a large scale and 2) the severe vegetative and floral phenotype abnormalities caused by constitutive hypomethylation make crossing technically difficult and unreliable. To be agriculturally useful, it will be necessary to engineer plants that have a WT vegetative and floral phenotype but produce seed that is smaller or larger than normal when self fertilized. Model 1 and 2 (**1.4.2.6**) predict that seed size can be altered by tissue-specific suppression of maintenance DNA methyltransferases. Both models predict that plants suppressing maintenance DNA methyltransferases during carpel development will produce seed that is larger than normal. Conversely, the models predict that plants suppressing maintenance DNA methyltransferase activity during anther development will produce seed that is smaller than normal. It is therefore proposed that an approach to suppress maintenance DNA methyltransferases tissue-specifically could be used to engineer plants that have a WT vegetative and floral phenotype and produce seed that is smaller or larger than normal when self fertilized.

The reciprocal parent-of-origin-specific effect on seed size resulting from the inheritance of hypomethylated genomes have so far only been demonstrates in *Arabidopsis*. To assess whether this approach can be used to alter seed size in agriculturally important species it is necessary to test this approach in plant species other than *Arabidopsis*. As these reciprocal effects on seed size are caused by the altered expression of imprinted genes and imprinting is prevalent across the angiosperm, it is predicted that reciprocal parent-of-origin-specific effects on seed size will be induced via the inheritance of hypomethylated genomes in agriculturally important plant species.

To alter DNA methylation, and thus seed size, in a variety of plants it would be advantageous to develop an approach that can suppress DNA methyltransferase activity in any plant, independent of its species. Eukaryotic DNA methyltransferases have retained

conserved sequence motifs from their prokaryotic ancestry. It is therefore hypothesised that a silencing approach designed to target the suppression genes encoding these motifs could be used to suppress maintenance DNA methyltransferase activity in a broad spectrum of plant species

1.6 *Thesis aims and objectives*

The aims of the work reported in this thesis and the objectives used to achieve them are summarised below.

1: ***To determine whether MET2a, MET2b and MET3 play a role in genomic imprinting.***

The functionality of *MET2a*, *MET2b* and *MET3* was investigated using *in silico* methods and via phenotypic and DNA methylation analysis of *MET1* family T-DNA insertion lines. Subsequently *MET1* family T-DNA insertion lines were analysed for altered imprinting.

2: ***To develop a method to suppress MET1 tissue-specifically to 1) investigate when MET1 is required for imprinting and 2) alter seed size***

A *MET1*::*GFP* reporter was developed and used to map *MET1* expression during anther and carpel development and the male and female gametophyte generation. An approach to induce *MET1* suppression via the RNAi pathway was developed and tools to signal hypomethylation and altered imprinting were analysed. Subsequently attempts were made to suppress *MET1* tissue-specifically by the production of transgenic lines carrying promoter-specific *MET1*-suppression constructs. These lines were analysed for tissue-specific hypomethylation and altered imprinting using the tools referred to above.

3: ***To determine whether suppression of maintenance DNA methyltransferase activity alters seed sizes in tobacco and whether a transgene designed to suppress*** **MET1** ***in*** **Arabidopsis** ***(*****AtMET1*****) can induce*** **MET1** ***suppression in tobacco (*****NtMET1*****).**

Seed size analysis was preformed on the progeny from reciprocal crosses between hypomethylated *NtMET1*as lines and WT plants in tobacco. Transgenic tobacco lines carrying constructs designed to induce AtMET1 and NtMET1 expression were produced and analysed for hypomethylation.

4: ***To investigate preliminary observations of vegetative phenotype differences between [WT X met1-9] and [met1-9 X WT] heterozygotes***

The vegetative phenotype of [WT X *met1-9*] and [*met1-9* X WT] heterozygotes was compared. A suggestion that ectopic *FWA* expression is responsible for phenotype differences between reciprocal *met1-9* heterozygotes was subsequently tested by analysing the phenotype of [WT X *met1-9fwa-3*] and [*met1-9fwa-3* X WT] double heterozygous loss-of-function mutants.

Chapter 2 – *Material and methods*

2.1 *Materials*

2.1.1 *Plant material*

All *Arabidopsis* plants were columbia-0 (Col-0) unless otherwise stated. *MET1* family T-DNA insertion lines were obtained from the SALK database at the Nottingham Arabidopsis Stock Centre (NASC). *met1-9, met2a-1, met2b-1* and *met3-1* lines are SALK lines N576522, N510893, N548436 and N599592 respectively. The *fwa-1* gain-of-function (Koornneef, M et al. 1991) and the *fwa-3* loss-of-function lines were donated by Tetsuji Kakutani (National Institute of Genetics, Japan) via Wim Soppe (Max Planck Institute for Plant Breeding Research, Cologne). The *MEA*::*GUS* reporter (Spillane, C. et al. 2004) was donated by Ueli Grossniklaus (Institute if Plant Biology, Switzerland) and *FWA*::*GFP* reporter (Kinoshita, T. et al. 2004) was donated by Tetsu Kinoshita (National Institute of Genetic, Japan). *35S*::*NtMET1*as tobacco lines were donated by Yuka Nakona (Nara Institute of Science and Technology, Japan).

2.1.2 *Plant growth media*

Plant growth media used for *Arabidopsis* germination consisted of an autoclaved solution of Murashige & Skoog Medium with Gamborgs Vitamins (Sigma) and 1% sucrose, adjusted to pH5.7. Media used for tobacco germination consisted of an autoclaved solution of ½ Murashige & Skoog Medium with Gamborgs Vitamins and 1% sucrose, adjust to pH5.7. MSD4x2 media used for tobacco tissue culture consisted of an autoclaved solution of Murashige & Skoog Medium Basal Medium (Sigma) and 3% sucrose. 0.8% agar was added to all of the above if solid media was required.

2.1.3 *Bacterial strains*

TransforMax™ EC100 Electrocompetent *E. coli* (Epicentre) were used for cloning and GV3101 *A. tumefaciens* were used for plant transformation. This agrobacterial strain harbors a non-oncogenic Ti plasmid (pGV301) (Van Larebeke, N. et al. 2008).

2.1.4 *Bacterial growth media*

E. coli and *A. tumefaciens* were grown on LB and 2YT media respectively. LB media consisted of an autoclaved solution of 5g/L NaCl, 5g/L yeast extract (Fischer) and 10g/L trypton (Fischer), adjusted to pH 7.2 with NaOH. 2YT media consisted of an autoclaved

solution of 5g/L NaCl, 5g/L yeast extract and 16g/L trypton, adjusted to pH 7.2 with NaOH. For solid media 1.5 % granulated agar (Difco) was added.

2.1.5 *Plasmids*

Plasmids used for cloning include pGEM-T Easy vector (Promega), pBI (*dme* NLS-*GFP*) (Choi, Y. et al. 2002) and pFGC4591. pBI (*dme* NLS-*GFP*) was kindly donated by Robert Fisher (University of California, USA) and pFGC4591 was obtained from the Chromatin Database (www.chromdb.org/).

2.1.6 *Oligonucleotides & Sequencing*

Gene specific oligonucleotides and linkers were synthesised by Invitrogen or Sigma-Aldrich. All DNA sequencing reactions were performed by Lark Technologies DNA sequencing (Essex, UK).

2.2 *Methods*

2.2.1 In silico *DNA and amino acid sequences analysis*

DNA and amino acid sequence alignments were performed using GeneDoc Version 2.6.002. Ka/Ks substitution ratio analysis was kindly performed by Prof. L. Hurst (University of Bath, UK).

2.2.2 *Plant Growth*

2.2.2.1 *Seed germination and plant growth in soil*

Arabidopsis seed was stratified in a solution of 0.15% agar technical (Oxoid) at 4ºC for 3-4 days prior to sowing on F2 compost (Levingtons). Each liter of soil was pre-treated with 100ml of 0.02% intercept 70WG solution (Scotts). *Arabidopsis* plants were grown in controlled environment rooms with a day length of 16hr, a temperature of 23°C during the day and 18°C at night and 70% humidity. Tobacco seed was sown directly on untreated compost and tobacco plants were grown in a heated glass house with a temperature range of 22-40°C.

2.2.2.2 *Seed germinated on plant growth media*

Seed was surface sterilised by shaking for 5min in 70% ethanol followed by 5min in 50% bleach plus 0.05% Tween 20 (Bio-Rad). Seed was then washed 6 times in ddH_2O, re-suspended in 0.15% agar technical and transferred to Petri dishes containing plant growth

media. Petri dishes were sealed with parafilm and, if necessary, were stratified 4ºC for 3-4 days before transferring to growth rooms. After approximately 2 weeks seedlings were transferred to soil and grown as described above.

2.2.3 *Cross pollination and seed collection*

2.2.3.1 *Cross Pollination*

Anthers of the seed parent were dissected from floral buds one day prior to anthesis. The seed parent was pollinated three days later by dabbing mature pollen from the pollen-parent onto the mature stigma. Occasionally male sterile A9 barnase plants were used as seed parents. These plants were pollinated without emasculation.

2.2.3.2 *Restricted seed set*

Restricted seed set was used to restrict siliques production. Once a defined number of siliques were produced on the primary inflorescence stem, the primary inflorescence meristem, all auxiliary flowering shoots, secondary inflorescence stems and all others siliques and flowers were removed.

2.2.3.3 *Seed collection*

Matures seeds were collected when siliques desiccated and were stored in 1.5ml tubes with pierced lids to allow further drying. Occasionally seed was stored in a sealed container with silicon gel (Sigma) to accelerate drying.

2.2.4 *Production of double mutants*

MET2a and *MET2b* are both located on chromosome 4 (at 8.144Mb and 5.762Mb respectively), therefore the production of a *met2a-1met2b-2* double mutant relied on a crossing over and recombination event between these two loci. *met2a-1met2b-2* homozygous double mutants were made by crossing *met2a-1* and *met2b-1* homozygotes to produce an F1 generation heterozygous for both T-DNA insertions. F2 plants was genotyped using PCR to identify those transmitting the appropriate recombination event, which were homozygous for one T-DNA insertion and heterozygous for the other. *met2a-1met2b-2* homozygous double mutants were identified by genotyping the F3 generation derived from these plants.

met1-9fwa-3 homozygous double mutants were made by crossing *met1-9* and *fwa-3* homozygotes to produce an F1 generation heterozygous for both T-DNA insertions. As *met1-9* homozygous seed was known to be small (Chapter 3, Section **3.2.5.2**), small F2 seed was sown to select for *met1-9* homozygotes in the F2 generation. Genotyping using PCR was then used to identify F2 *met1-9fwa-3* homozygous double mutants.

2.2.5 *Genotyping and gene/transgene/transcript detection using PCR*

2.2.5.1 *DNA extraction*

Rapid DNA extraction from plant tissue was performed using a protocol adapted from (Edwards, K. et al. 1991). Approximately 0.75cm² of fresh leaf tissue, or an equivalent volume of another tissue, was ground in 1.5ml tubes containing 450µl of DNA extraction buffer (an autoclaved solution of 200mM Tris-HCl pH 7.5, 25mM EDTA, 25mM NaCl) using pestle grinders and acid washed glass beads (Sigma). The suspension was centrifuged at 14,000rpm for 10min and the supernatant was carefully transferred to a fresh tube. The centrifugation step was then repeated and 350µl of supernatant was transferred to a fresh 1.5ml tube and mixed with an equal volume of isopropanol. The mix was incubation at room temperature for 15min to allow DNA to precipitate and then DNA was pelleted by centrifugation at 14,000rpm for 10min. The pellet was washed twice in 70% ethanol and air dried before resuspending in 80µl ddH_20.

2.2.5.2 *PCR*

For genotyping and the detection of genes, transgenes or transcripts, PCRs was carried out using Go Taq Flexi DNA Polymerase (Promega). Reaction mixes consisted of DNA template, 5X Green GoTaq Flexi Buffer (Promega), 1.5mM $MgCl_2$, 0.2mM dap, dCTP, dGTP and dTTP (Promega), 0.4µM forward and reverse primers and 0.175u/µl Go Taq Flexi DNA Polymerase. A typically 25µl reaction contained 2µl of DNA or mRNA template, 5µl of 5X Green Go Taq Flexi Buffer, 1.5µl of 25mM $MgCl_2$, 0.5µl of 10mM dNTP mix, 1µl of 10mM forward and reverse primers, 0.175µl of 5u/µl Go Taq Flexi DNA Polymerase and 13.875µl ddH_20. All reactions were carried out using a MJ Research PTC-200 Peltier Thermal Cycler. A typical amplification programme had an initial denaturation step at 94°C for 10min followed by 32 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 60s and then a finished step of 72°C

for 10min. For specific genotyping reactions, primer sets, annealing temperature and extensions times are detailed in **Table 2.1** and primer sequence are detailed in **Table 2.2.**

Table 2.1 PCR conditions used for genotyping *Arabidopsis* T-DNA insertion lines

PCR Description	Fragment amplified	Primer names	Annealing Temp. °C	Extension Time (s)	Product Size (bp)
met1-9 genotyping	Genomic	LSB5.F, LSB6.R	65	50	370
	T-DNA	lba1, LSB6.R	65	50	~520
met2a-1 genotyping	Genomic	Stm2a893F, Stm2a893R	47	50	587
	T-DNA	lba1, Stm2a893R	48	80	~890
met2b-1 genotyping	Genomic	Stm2b436F, Stm2b436R1	63	50	554
	T-DNA	lba1, Stm2b436R1	63	50	~800
met3-1 genotyping	Genomic	N599592F, N599592R	62	50	772
	T-DNA	N599592F, lba1	62	50	~740
fwa-3 genotyping*	Genomic & T-DNA	JL202, TKF2, FWA2	44	45	~660 ~840

* *fwa-3* genotyping was performed using a multiplex PCR

Table 2.2 The sequence of primers used for genotyping *Arabidopsis* T-DNA insertion lines

Primer Name	Primer Sequence (5' → 3')
Lba1	TGGTTCACGTAGTGGGCCATCG
LSB5.F	AATCTGAACACCTGCCTCACAGGATGC
LSB6.R	TTATACATCAGCAACAGAAGAAAAAACAGACG
Stm2a893F	AATTATCTTATATGAACTAAAG
Stm2a893R	GACTTTTACACCTCCTTCTCCT
Stm2b436F	ACGATTGCCGTAAACCAGCTTCT
Stm2b436R1	ACCTCTTAGTAACCGTTAGAGCAC
N599592F	TGCTAAACTACTGCAAGAGGAAGAACATAT
N599592R	AATGTGTGTTTCGTGGAATGTTGTTATCT
JL202	CATTTTATAATAACGCTGCGGACATCTAC
TKF2	GTGACTCTGGTCAAGACT
FWA2	GTTGGTAGATGAAAGGGTCGAGAG

2.2.5.3 *Agarose gel electrophoresis*

Gels were made by boiling a mixture of 1xTAE (40mM Tris-acetate, 1mM EDTA) with 0.8-1.5% agarose (Invitrogen). While liquid, 4μl of 10 mg/ml EtBr was added per 100ml of gel and gels were set in Bio-Rad trays. Electrophoresis was performed in a Sub-Cell tank (Bio-Rad) with a Bio-Rad Powerpac 300 power supply, usually set at 100V, and using

a 1xTAE running buffer with EtBr added. DNA bands were visualized on a transilluminator with 70 % UV light at 254nm.

2.2.6 *Amplification and purification of DNA fragments for cloning or sequencing*

2.2.6.1 *PCR using proof reading* Taq

For the amplification of fragments for cloning or sequencing PCR was performed using KOD HiFi DNA Polymerase (Novagen). Reaction mixes consisted of DNA template, 10X Buffer#1 for KOD HiFi DNA Polymerase (Novagen), 1mM $MgCl_2$, 0.2mM dNTP mix (Novagen), 0.4µM forward and reverse primers, 2.5U/µl KOD HiFi DNA Polymerase. Typically 50µl reactions were set up containing 5µl of genomic DNA, 5µl of 10X Buffer#1, 2µl of 25mM $MgCl_2$, 5µl of a 2mM dNTP mix, 2µl of 10mM of forward and reverse primers, 0.4µl of KOD HiFi DNA Polymerase and 28.6µl ddH_20. All PCRs were carried out using a MJ Research PTC-200 Peltier Thermal Cycler as described above. Amplification programmes were set according to the KOD HiFi DNA Polymerase manufacturer's instructions. For specific reactions primer pairs, annealing temperature and extensions times are detailed in **Table 2.3** and primer sequence are detailed in **Table 2.4**.

Table 2.3. PCR conditions used for the amplification of fragments for cloning

PCR Description	Primer names	Annealing Temp. °C	Extension Time (s)	Product Size (bp)
LMET1 promoter	LMET1F, LMET1 F	50	40	3567
SMET1 promoter	SMET1F, SMET1 F	52	40	2527
MET1-RNAi IR fragment	LSB56.F, LSB57.R	61	30	215
AP3 promoter	AP3F, AP3R	50	60	1761
SHP2 promoter	SHP2F, SHP2R	63	60	1558
APG promoter	APGF, APGR	59	30	451
At2g20070 promoter	At2g20070F, At2g20070R	42	30	440
AtMET1-RNAi IR fragment	AtMET1.2F, AtMET1.2R	50	0	352
NtMET1-RNAi IR fragment	NtMET1.2F, NtMET1.2R	55	0	352

Table 2.4. The sequence of primers used for the amplification of fragments for cloning Linkers highlighted in red. Restriction sites underlined

Name	**Sequence (5' → 3')**[a]	Restrictions sites
SMET1 F	AAAGTCGACCTCTGTAGATCGTGCATTATCG	*Sal*I
SMET1 R	AAATCTAGATTTCAAAATCCCTAGTTTCAAAATC	*Xba*I
LMET1 F	AAAGTCGACAAAAAACGGACCCGATAACC	*Sal*I
LMET1 R	AAATCTAGATTCCACCCTAAGAAAAGTAAG	*Xba*I
LSB56.F	AAATCTAGAGGCGCGCCGAAGAGTAGTAAGATTGACAAGCC TCTG	*Xba*I, *Asc*I
LSB57.R	AAAGGATCCATTTAAATAACCTGAGCTGCTGTTTTTTCTGGG	*BamH*I, *Swa*I
AP3F	AAAGAATTCAAGAATTATAGTAGCACTTGTTG	*EcoR*I
AP3R	AAACCATGGCATATTCTTCTCTCTTTGTTTAATC	*Nco*I
SHP2F	AAAGAATTCACGAAAGTCAATCAAAAGACCTACC	*EcoR*I
SHP2R	AAACCATGG TTACTCGCACCACCCTCCATTTC	*Nco*I
APGF	AAAGAATTCGATCGAATCCATCTCATTCCAAC	*EcoR*I
APGR	AAACCATGGGATCGCTTCATGGTTTTACTACAAG	*Nco*I
At2g20070F	AAAGAATTCTGAACAATTATTATGCTTAA	*EcoR*I
At2g20070R	AAACCATGGAAACCTACTCACTTATATA	*Nco*I
AtMET1.2F	AAACCCGGGGGCGCGCCCTAAAGAGATTCGTCTGGC	*Xma*I, *Asc*I
AtMET1.2R	AAAGGATCCATTTAAATTTCATACCAGAAAATCCCTGAC	*Swa*I, *BamH*I
NtMET1.2F	AAATCTAGAGGCGCGCCCTCAACAGAATCGTTTGGC	*Xba*I, *Asc*I
NtMET1.2R	AAAGGATCCATTTAAATTTCATTCCAGAAAACCCCTGAC	*Swa*I, *BamH*I

[a] Linkers highlighted in red. Restriction sites underlined.

2.2.6.2 *Gel purification of DNA fragments*

DNA fragments were separated using gel electrophoresis and recovered using the Wizard SV Gel and PCR Clean-UP System (Promega) as described by the manufacturer's instructions. If necessary, DNA fragments were concentrated using Pellet Paint NF Co-Precipitant (Novagen) as described by the manufacturer's instructions.

2.2.7 *Molecular cloning and bacterial transformation*

2.2.7.1 *A-tailing*

A-tailing was required when cloning into pGEMT. 10µl reactions were set up consisting of 7µl of gel purified DNA fragment, 1µl of 10X PCR Buffer (Sigma), 1µl of 0.2mM dATP and 1µl of Taq DNA polymerase (Sigma). The reaction was heated for 15 min at 70°C in a thermal cycler.

2.2.7.2 *Ligation*

Ligation of plasmids was performed using T4 DNA ligase (Promega) and 2x Rapid Ligation buffer (Promega) according to the manufacturer's instructions. Typically a 1:3 molar ratio of vector to insert was used and reactions were incubated at 4°C overnight.

2.2.7.3 *Transformation to* E. coli

Plasmid was transformed *E. coli* by electroporation. 30-50µl of cells were thawed on ice and 1-3µl of plasmid or ligation mix was added and mixed by tapping gently. The mixture was then transferred to a pre-cooled 0.2cm electroporation cuvette (Bio-Rad). Electroporation was performed using a Gene PulsarTM and pulse controller until (Bio-Rad) set at a field strength of 12.5V/cm, a capacitance of 25 µF and a resistance of 400ohms. Immediately after electroporation 1ml of pre-warmed LB was added. The liquid culture was then incubated at 37°C for 1hr with shaking and then 100µl and 200µl were plated onto LB plates with appropriate antibiotics (25µg/ml kanamycin or 100µg/ml carbenicillin) and incubated at 37°C overnight. For transformation of plasmids with a pGEMT backbone, positive colonies were identified using blue/white screening according to the manufacture's instructions (Promega). For all transformations colonies were screened from the presence of the correct plasmid using colony PCR (**2.2.7.4**) and the checked by the restriction digestion of plasmid minipreps (**2.2.7.5**).

2.2.7.4 *Colony PCR*

Colonies were picked up using sterile tips and resuspended in 10µl ddH_2O. To ensure the colony was maintained a small amount of the suspension was spotted on to a fresh LB plate and incubated at 37°C for approximately 8hrs. The remaining suspension was heated for 10min at 95°C to rupture cells and cell debris was then pelleted by centrifugation. 5µl of supernatant was used as template DNA for PCR reaction as described in **2.2.5.2**.

2.2.7.5 *Plasmid DNA purification and restriction digestion*

To amplify plasmid from bacterial, a single colony was picked up using a sterile tip and resuspended in 5µl liquid LB plus antibiotics. Liquid culture was growth over-night at 37°C with shaking. Plasmid DNA was then purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) as described by the manufacture's instructions.

Restriction digestion was used to check plasmid structure and to cut transgene from plasmid, or linearise plasmid, in preparation for cloning. Digestion reactions using the appropriate restriction enzymes were set up according to the manufacture's instructions. All reactions were incubated overnight at 37°C. Plasmid fragments were then separated by gel electrophoresis (**2.2.5.3**). Fragmented required for cloning were subsequently gel purified (**2.2.6.2**).

2.2.7.6 *Preparation of electrocompetent* A. tumefaciens

A. tumefacines GV3101 cells were streaked from a glycerol stock on to LB plates containing 100µg/ml rifampicin, 25µg/ml gentamycin and 100µg/ml carbenicillin and grown for 48hrs at 28°C. 10ml of liquid 2YT plus antibiotics (as above) was inoculated with single colony of *A. tumefacines* and this starter culture was incubated overnight at 28°C with shaking. 2 x 50ml of liquid 2YT plus antibiotics (as above) were inoculated with 5ml of the *A. tumefacines* starter culture and cultures were again grown overnight at 28°C with shaking. Cells were harvested by centrifugation at 7500rpm for 5min at 4°C. The pellet was responded in 50ml of 10% (v/v) glycerol chilled on ice. The centrifugation and resuspension steps were repeated with 20ml, 5ml and 1ml of 10 % glycerol. After a final centrifugation the pellet was resuspended in 200µl 10% glycerol, snap frozen in liquid nitrogen and stored at -80 °C

2.2.7.7 *Transformation into* A. tumefaciens

Transformation in to *A. tumefaciens* was performed using electoporation as described for E. *coli* (**2.2.7.3**). However, for *A. tumefaciens* transformation the 1ml liquid culture was incubated at 28°C for 2hrs with shaking before 100µl and 200µl were plated onto LB plus antibiotic (100µg/ml rifampicin, 25µg/ml gentamycin and 25µg/ml kanamycin). Plates were then incubated at 28°C for approximately 36hrs.

2.2.8 *Plant Transformation*

2.2.8.1 *Preparation of* A. tumefaciens *culture*

50 ml of 2YT plus antibiotics (100µg/ml Rifampicin, 25 µg/ml Gentamycin and 25 µg/ml Kanamycin) was inoculated with a colony of transformed *A. tumefaciens* and liquid culture was grown overnight at 28°C with shaking. At this point, PCR was used to check for the transgene in the *A. tumefaciens* culture. Cells from 500µl of culture were pelleted by

centrifuging at 8500rpm for 5min. The supernatant was poured away and the cells were resuspended in 100 µl H_2O. To denature the cells, 50 µl of the suspension was heated to 95% for 10min and then the cell debris was pelleted by centrifugation. 5 µl of the supernatant was then used as DNA template for PCR as described in **2.2.4.2.** The remaining 50ml culture was then used to inoculate 500ml 2YT plus antibiotics (as above) and cultures were again grown overnight at 28°C with shaking.

2.2.8.2 *Floral dipping*

500ml of *A. tumefaciens* liquid culture was centrifuged at 7500rpm for 15min to pellet cells. The supernatant was poured away and cells were resuspended in 500ml of 5% sucrose with 0.036% Silwett L-77 (Lehle Seeds). The cell suspension was poured into a tilted seed tray and floral were dipped for 10s with gentle agitation. Plants were then watered, covered with an autoclaved bag and kept in the shade for approximately 24hrs before returning to their normal growth environment.

2.2.8.3 *Selection of transformants*

If plants were transformed with plasmid carrying a kanamycin resistance gene, T1 lines were selected by germination on plant media containing 50 µg/ml of kanamycin. If plants were transformed with plasmid carrying a BASTA resistance gene, T1 lines were selected by watering seedling with 64mg/l BASTA (427.5µl/l of a 150g/l stock (AgrEvo))

2.2.9 *Tobacco leaf disk transformation and regeneration of plants from tissue culture*

2.2.9.1 *Preparation of* A. tumefaciens *culture*

5ml of 2YT plus antibiotics (100µg/ml Rifamipic, 25 µg/ml Gentamycin and 25 µg/ml Kanamycin) was inoculated with a single colony of transformed *A. tumefaciens* and the culture was grown overnight at 28°C with shaking. A this point PCR was then used to check for the transgene presence in the culture as described in **2.2.8.1**. The culture was then diluted 1:20 by a solution of Murashige & Skoog Media (Sigma M5519) and 3 % sucrose, adjusted to pH5.8.

2.2.9.2 *Leaf disk transformation and the regeneration of transformants*

Leaf disk transformation was performed in a laminar flow hood using aseptic technique. Young tobacco leaves were sterilized by submerging in 10% bleach for 15 min and then

washed four times in autoclaved tap water. 0.5-1cm^2 squares were cut from the leaves and transferred into Petri dishes containing 20ml of *A. tumefaciens* culture. Care was taken to ensure that all leaf disk edges were submerged in culture. The Petri dish lids were replaced and the dishes were incubates at room temperature for 20min. Leaf disks were then carefully transferred onto MSD4x2 plates containing 0.1 mg/l NAA and 1mg/l BAP to promoter shoot growth. A plate were sealed with parafilm and incubated at 24-26°C in low light (2000-4000 lux) for 48hrs. Leaf disks were then transferred to MSD4x2 plates containing 0.1 mg/l NAA, 1mg/l BAP and 4mg/l BASTA to select for transformants. Again the plates were sealed with parafilm and incubated at 24-26°C at low light intensity. After approximately one week, shoots were dissected from explants and transferred into jars contained solid hormone free MSD4x2 media with 4mg/l BASTA to encourage roots growth of transformants. Once roots had established plantlets were transferred to soil.

2.2.10 *Expression analysis by RT PCR*

2.2.10.1 Total *RNA extraction*

Approximately 30 μg of fresh plants material was frozen on dry ice or in liquid nitrogen and ground using a flame sterilised pestle and mortar. Smaller sample tissue were ground in a 1.5 ml tube using a pestle grinder and acid washed glass beads. RNA was extracted from using the SV Total RNA Isolation System (Promega). If necessary RNA was given a second DNAase treatment using the RNAase-Free DNAase set (Qiagen) and then concentrated using the RNeasy MinElute Cleanup Kit (Qiagen). RNA was quantified using the S2000 UV/Vis Spectrophotometer (WPA).

2.2.10.2 *cDNA synthesis*

cDNA was synthesised from 1μg total RNA using the Reverse-iT 1st strand Synthesis Kit (ABgene). PCR was then performed using the cDNA as a template (**2.2.5.2**).

2.2.11 *Southern analysis of genomic DNA*

2.2.11.1 *Extraction and digestion of* Arabidopsis *DNA*

Approximately 12 leaves were ground in liquid nitrogen with 1μl of 48.7% mercaptoethanal and approximately 2% PVPP w/w. The ground tissue was added to 300μl of DNA extraction buffer (50mM Tris-HCl pH8, 50mM Sod. EDTA pH 8, 250mM NaCl and 15% sucrose added post-autoclaving) and the solution was incubated at 4°C for 15min

before centrifuging at 5000rpm for 5min. The pellet was collected and carefully suspended in 300µl of 10:1 Tris:EDTA. 200µl of 20% SDS was added and the solution was incubated at 70°C for 30min. The solution was then left to cool room temperature before 250 µl of 5M Potassium acetate pH 5.2 was added. The solution was mixed gently by inverting and incubates on ice for 2hrs. The suspension was then centrifuged at 4°C for 10min at 12000rpm and the supernatant was collected and transferred to a fresh tube. DNA was precipitated by adding 500µl of isopropanol and mixed by inverting. The suspension was then incubated on ice for 10min and then DNA was pelleted by centrifuging for 10min at 12000rpm. The pellet was washed twice is 70% ethanol, air dried and dissolving in 40-50µl of 10:1 TE. 3-4 µl of DNA was run on a 1.2% agarose gel and quantified by eye. 4.5µg of DNA was then digested with either *Hap*II or *Msp*I according to the manufacture's instructions.

2.2.11.2 *Extraction and digestion of* Tobacco *DNA*

6 cm^2 of leaf tissue was ground in liquid nitrogen and added to 600µl DNA extraction buffer (1.4M NaCl, 20mM EDT, 100mM Tris-HCl pH 8, 3% CTAB w/v and 1% β-mercaptoethanol v/v), mixed and incubated at 65°C for 10min. The solution was then allowed to cool to room temperature before DNA was extracted using 600µl of chloroform-isoamyl alcohol. The solution was mixed then centrifuged at for 10min at 14000rpm. The upper aqueous layer was then transferred to a new tube and the centrifugation step was repeated. Again the upper aqueous layer was transferred to a new tube and 1/10 Sodium acetate and an equal volume of propanol was added to precipitate DNA. The solution was incubated at room temperature for 2hrs and then DNA was precipitated by centrifugation for 15 min at 14000rpm. The DNA pellet was then dissolved in 35µl of 10:1 TE. 3-4 µl of DNA was run on a 1.2% agarose gel and quantified by eye. 5µg of DNA was then digested with either *Hap*II or *Msp*I according to the manufacturer's instructions.

2.2.11.3 *Transfer of DNA to the nylon membrane*

DNA fragments were separated by electrophoresis on a 0.95% agarose gel made with 1X TAE minus EtBr, using a 1X TAE running buffer plus EtBr. The gel was then incubated in depurination solution (1.5M NaCl and 0.5M HaOH) for 20 min with gentle agitation. The gel was then washed in ddH_2O and incubated in denaturation solution (250mM HCl

prepared fresh) for 45min with gentle agitation. The gel was then washed three times in ddH_2O and incubated in neutralisation solution (0.5M Tris-HCl pH 7.5 and 1.5M NaCl) for 30min with gently agitation before washing again in ddH_2O. DNA was transferred to a nylon membrane (Genetix) with 10X SSC using self constructed bolting apparatus which were set up as follows. An oblong tray was filled approximately 2.5cm deep with 10X SSC (a 1:2 dilution of a 20X SSC stock: 3M NaCl 0.3M Sodium Citrate adjust to pH 7.0). Two large gels trays were rested across the width of the tray and Whatman 3MM paper, pre-wetted in 10X SSC, was draped over the gel traps so that opposite ends of the Whatman paper were submerged in the 10X SSC. The Whatman paper was carefully smoothed out using a rolling pin to ensure that no air bubbles were caught. The top right corner was cut from the gel and then the gel was laid facing down on the Whatman paper and again smoothed out to remove air bottles. A piece of nylon membrane, handled with forceps, was cut to the size of the gel and the top right corner of the nylon membrane was removed. The membrane was the laid on top of the gel so that the cut corners were aligned. Again the stack was smoothed to remove air bubbles. Parafilm was then laid over exposed Whatman paper around the edge of the gel to restrict 10% SSC rise though the gel. 3 pieces of 10% SSC wetted Whatman paper cut to size were laid on top of the membrane and then a further 15-20 dry pieces of Whatman paper, one box of facial tissues and several cm of blue roll were laid on top. Finally a 1kg weight was balanced on the stack. The stack was left over night to allow DNA transfer. DNA then fixed to the membrane using UV crosslinking at 24J/cm^2 and then at 20x 0.1J/cm^2.

2.2.11.4 *Preparation of dCTP labeled probe*

The probes were labeled with [^{32}P] dCTP using the Prime-It II Random Primer Labeling Kit (Stratgene). The probe was cleaned by passing through a Sephadex G50 column. The column was made by pressing glass wool into a 1ml syringe. Sephadex G50 was then added to the 0.8ml line of the syringe. The column was washed with 3 X 150µl STE buffer (100mM NaCl, 20mM Tris-HCl pH 7.5 and 10mM EDTA). The labeled probe was then made up to 150µl with STE buffer and washed though the column.

2.2.11.5 *Hybridisation*

Prior to hybridisation the membrane was incubated in a roller at 65°C with 50ml of pre-warmed Church buffer (0.5M phosphate buffer pH7.2, 7% SDS. 1mM EDTA and 1%

BSA) for 2-4hrs. This Church buffer was then removed and 12ml of fresh buffer containing the labeled probe was added. The member and probe were incubated in a roller at 65°C overnight. The member was then washed twice with 2X SSC by rolling for 10min at room temperature. The washing step was then repeated with 2X SSC plus 0.1% SDS and twice with 2X SSC plus 1% SDS. The membrane was then wrapped in Saran wrap and exposed to X-ray film for 4-36hrs.

2.2.12 *Histochemical localization of GUS activity*

Tissue was submerged in GUS staining buffer (100mM KPO_4, 1mg/ml X-Gluc, 0.25mM $K_3Fe(CN)_6$, 0.25mM $K_4Fe(CN)_6$ and 0.1% Triton X-100) in the wells of a 96 well plates. Plates were then foil wrapped and incubated at 37°C for overnight. Tissue was the imaged using microscopy.

2.2.13 Microscopy

All plants maternal, except pollen, was mounted on glass slides in 10 % glycerol. Pollen was mounted in 10 % sucrose. Cover slips were sealed onto slides using nail polish. DIC and florescence microscopy was performed using a 90i Eclipse microscope (Nikon). Confocal microscopy with performed using a Nikon C1 confocal microscope system with the 90i Eclipse microscope. GFP was visualised using a 488nm Argon Laser and a 515-530nm filter.

2.2.14 Image capture and processing

Photographs of whole plants were taken using a Nikon Coolpix 4500 digital camera (Nikon). Microspore images were captured with Nikon Digital Sight DS-U1 and NIS-Elements F software. All images were processed using Photoshop Elements (Adobe).

2.2.1.5. Statistical analysis

All statistical analysis was performed using Minitab 15.1.0.0. Each data set was tested for normality and homogeneity of the variance using the A.on-Darling Normality Test and the F-test respectively. Parametric data was analysed using the T-test or ANOVA and non-parametric data was analysed using the Mann-Whitney U test or the Kruskal-Wallis test. Phenotype variation between *MET1* family T-DNA insertion lines was analysed using the Mood's Median test instead of the Kruskal-Wallis test because it gives an output that immediately identifies the outlier.

Chapter 3 – *The role of the* MET1 *gene family in genomic imprinting*

3.1 *Introduction*

The involvement of MET1 in plant genomic imprinting is indicated from the complementary seed-size phenotypes produced from reciprocal *MET1*as X WT crosses; [*MET1*as x WT] seed exhibits endosperm over-proliferation characteristic of paternal excess, whereas [WT x *MET1*as] seed exhibits endosperm under-proliferation characteristic of maternal excess (Adams, S. et al. 2000, Scott, R. J. et al. 1998). These observations suggest MET1 plays a role in silencing of maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes.

In Chapter 1 (section **1.4.2.3**), it was argued that MET2a, MET2b and MET3 may also play a role in genomic imprinting. *MET2a*, *MET2b* and *MET3* are annotated putative maintenance DNA methyltransferases but their specific role has not been reported. The putative coding regions of *MET2a*, *MET2b* and *MET3* shares 78%, 78% and 70% nucleic acid sequence homology with *MET1* respectively. This sequence homology indicates that these genes may have similar functionality to *MET1* and therefore could play a role in controlling imprinted gene expression. The involvement of *MET1* in imprinting has been demonstrated predominantly from the study of hypomethylated *MET1*as lines and it is argued that *MET1*as expression may cause off-target suppression of other *MET1* family genes (Chapter 1 section **1.4.2.3**). Effects on imprinting caused by *MET1*as expression could therefore be partially due to *MET2a*, *MET2b* and/or *MET3* suppression.

The aim of the work described in this chapter was to determine whether *MET2a*, *MET2b* and *MET3* play a role in imprinting. The functionality of these genes was assessed using *in silico* analysis and the analysis of *MET1* family loss-of-function mutants. Subsequently, a putative role for MET2a, MET2b and MET3 in imprinting was investigated by testing for altered imprinting in *MET1* family loss-of-function mutants.

3.2 *Results*

3.2.1 In silico *analysis of* MET1 *family gene sequences and expression profiles*

In silico analysis of *MET2a*, *MET2b* and *MET3* genomic sequences and the putative amino acids sequences encoded by these genes was performed to assess whether they encode functional DNA methyltransferases. Additionally, publicly available microarray data was

analysed to determine the expression profile of these genes with the aim of providing clues to gene function.

3.2.1.1 *Sequence comparison of MET2a, MET2b and MET3 with MET1*

The putative amino acid sequences of MET2a, MET2b and MET3 was compared with that of MET1 with the aim of identifying putative functional domains known to be characteristic of or/and fundamental for the DNA methyltransferase activity. The putative amino acid sequence of MET2a, MET2b and MET3 was aligned with that of MET1 and the sequence of MET1 functional domains in was compared with the corresponding sequence of these other MET1 family members.

The far N-terminal of MET1 is rich in basic amino acids reduces which are suggested to constitute a nuclear localisation signal (NLS) (Finnegan, E. J. and Dennis, E. S. 1993). The corresponding region of MET2a, MET2b and MET3 is similarly rich is basic residues. Closer examination revealed that one motif, conserved in all MET1 family members, is characteristic of a bipartite NLS (**Figure 3.1a**); this consists of two regions of basic residues, the first has two basic residues and the second has at least three out of a five, separated by a spacer of more than four residues (Raikhel, N. 1992). Identification of these putative NLS indicates that, like MET1, MET2a, MET2b and MET3 are targeted to nucleus.

Down stream of the NLS, MET1 has a putative replication foci targeting sequence (RFTS) (Finnegan, E. J. and Dennis, E. S. 1993). In DNMT1, the mammalian maintenance DNA methyltransferase, this region, along with others, targets DNMT1 to the replication fork during S phase of the cell cycle, in which DNA replication occurs (Leonhardt, H. et al. 1992, Lui, Y. et al. 1998). DNA methyltransferase activity is believed to occur at the replication folk and thus targeting to this region is essential for DNA methyltransferases. MET1, MET2a and MET2b have high sequence homology at the MET1 RFTS suggesting that MET2a and MET2b also target the replication folk (**Figure 3.1b)**. In contrast, the putative amino acid sequence of MET3 has a 34 amino acid deletion within this region. Chimeric DNMT1 proteins with deletions in the RFTS fail to target the replication folk (Leonhardt, H. et al. 1992). A deletion within the RFTS of MET3 could therefore render this putative DNA methyltransferase inactive. The functionality of MET3 is further addressed in **3.2.1.2**.

Further downstream, MET1 has an acidic region of unknown function (Finnegan, E. J. and Dennis, E. S. 1993). A similar acid region is also present MET2a, MET2a and MET3 (**Figure 3.1c**). Downstream of this, MET1 has two bromo-adjacent homology (BAH) domains (Callebaut, J. et al. 1999). Putative BAH domains are also present in the corresponding region of MET2a, MET2b and MET3 and all seven BAH motifs were identified in the two BAH domains of each MET1 family member (**Figure 3.1d**). BAH domains have been identified in proteins involved in replication and transcriptional regulation and are suggested to facilitate protein-protein interaction (Callebaut, J. et al. 1999). The identification of BAH domains in MET2a, MET2b and MET3 again suggests that these proteins are targeted to the site of DNA replication and indicates that they may play a role in transcriptional regulation.

The amino terminal domains of MET1 is separated from the methyltransferase domain by a lysine-rich region (Finnegan, E. J. and Dennis, E. S. 1993). The corresponding region of MET2a, MET2b and MET3 is also lysine-rich (**Figure 3.1e**). A similar region bridges the two terminals of DNMT1 and is predicted to have functional significance (Finnegan, E. J. and Dennis, E. S. 1993).

DNA methyltransferases function by catalysing the transfer a methyl group from S-adenosyl-L-methionine (S-AdoMet) to carbon 5 of cytosine. The methyltransferase domain of MET1 has eight motifs conserved in all prokaryotic and eukaryotic methyltransferases. The function of the six most highly conserved motifs has recently been reviewed by Pavlopoulou, A. & Kossida, S (2007). Briefly, motif I and X are involved in S-AdoMet binding and a prolyl-cysteinyl doublet within motif IV reportedly forms the catalytic site. Motif VI plays a role in cytosine binding and motif VIII is suggested to make non-specific contacts with the cytosine and neutralise the negative charge of the DNA backbone. Finally, motif IX is reportedly involved in organising the target recognition domain, which in prokaryotes is located between motifs VIII and IX. All eight motifs present in MET1 have also been identified in the corresponding region of MET2a, MET2b and MET3 (**Figure 3.1f**), suggesting that these protein possess DNA methyltransferase catalytic function.

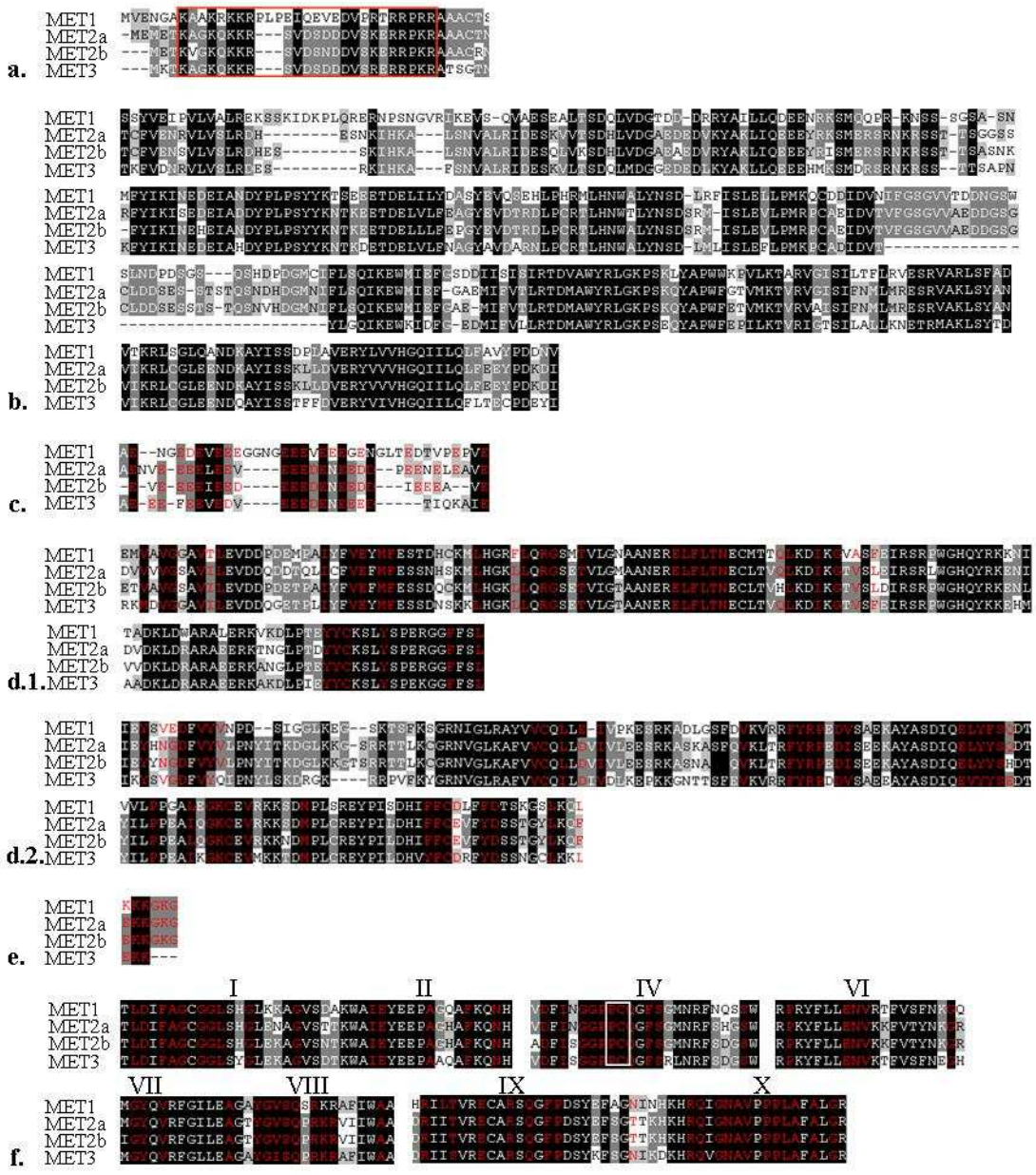


Figure 3.1 Putative domains and sequence motifs identified in MET2a, MET2b and MET3. a. Regions rich in basic amino acids. A red box highlights putative bipartite NLS. **b.** Putative RFTS. Note the fragment deletion in the MET3 sequence **c.** Acid rich regions. Red highlights glutamic acid (E) and aspartic acid (D) residues **d.** Putative BAH1 (**1.**) and BAH2 (**2.**) domains. Red highlights conserved residue positions within seven motif sequences (Callebaut, J. et al. 1999). **e.** Lysine rich regions. **f.** Conserved DNA methyltransferase catalytic motifs. Red highlights invariant amino acids positions or positions where only one of two or three possible amino acids occur in the prokaryotic methyltransferases used to identify the conserved motifs (Posfai, J. et al. 1989). The white box marks the prolyl-cysteinyl doublet predicted to be the methyltransferase catalytic site.

MET2a and *MET2b* apparently encode the same functional domains as *MET1* providing strong evidence that these genes encode maintenance DNA methyltransferases. However, the fragment deletion in the putative RLTS of *MET3* indicates this gene may encode a non-functional protein.

3.2.1.2 *Molecular evolutionary analysis of* MET1 *family genes*

All *MET1* family genes are reportedly derived from an ancestral *MET1*-like gene (Genger, R. K et al. 1999). Following such duplication events, the function of homologous genes can diverge or be conserved in response to positive selection and stabilizing selection respectively. The ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) between homologous genes is diagnostic of the selection to which genes have been subjected (Hurst, L. 2002). Ka/Ks ratio analysis was used to test whether selection had favoured the functional divergence or conservation of *MET1* family genes. The putative coding region of the *MET2a, MET2b* and *MET3* was aligned and compared with that of *MET1*. The Ka/Ks ratio for each gene pair was calculated for a window of 300 bps at the 5' end of the alignment. This window was then moved along the alignments by 3 nucleotides jumps and the Ka/Ks ratio was calculated for every window along the length of the genes.

In most windows the Ka/Ks ratio was considerably less than one (**Figure 3.2**). This indicates that nonsynonymous substitutions have been selected against and suggests the sequences have been subjected to stabilizing selection to preserve the amino acid sequence. Regions with very low Ka/Ks ratios, i.e. regions indicative of strong stabilizing selection, correlate with putative function domains (**Figure 3.2**). This provides strong evidence that the functional domains identified in *MET1* are conserved in all *MET1* family genes thus the functionality of *MET2a*, *MET2b* and *MET3* is likely to be similar to that of *MET1*.

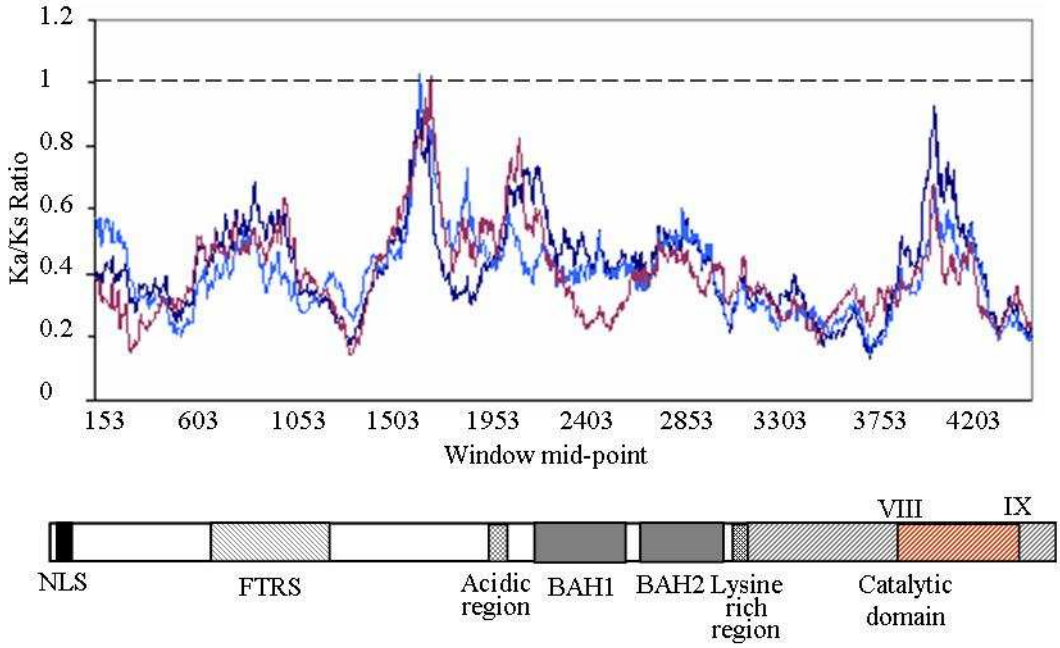


Figure 3.2 Overlapping sliding window analysis showing Ka/Ks ratios between *MET1* and *MET2a* (dark blue), *MET1* and *MET2b* (light blue), and *MET1* and *MET3* (purple). Below is a schematic of a *MET1* family gene showing the positions of putative functional domains in relation to the sliding window mid-points.

3.2.1.3 *Further analysis of the* MET3 *coding region*

MET3 is reported to encode a truncated protein in the *Arabidopsis* accession Columbia (Col-0) due to an in-frame stop signal at codon 65 and a deletion around codon 243 which causes a frame shift (Genger, R. K et al. 1999). As a consequence, MET3 is predicted to have little or no function in this accession (Finnegan, E. J. and K 2000, Genger, R. K et al. 1999). The mutations reported by Genger et al (1999) were identified within the *MET3* genomic sequence (www.arabidopsis.org). However, the positions of annotated *MET3* introns one and two indicate these mutations are spliced out and thus *MET3* encodes a full length transcript in the correct reading frame.

Interestingly, it was observed that annotated *MET3* introns one and two are unique to *MET3*; no introns are annotated in corresponding regions of *MET1, MET2a* and *MET2b* and *MET3* is the only *MET1* family gene to have annotated introns outwith a highly conserved pattern of introns (**Figure 3.3**). It was also observed that the DNA sequence of annotated *MET3* introns one and two is similar to the corresponding exonic regions of the other *MET1* family genes (**Figure 3.4**). Furthermore, annotated *MET3* intron two is located

within the putative RFTS and is responsible for the fragment deletion which could render MET3 inactive (See **3.3.1.1**).

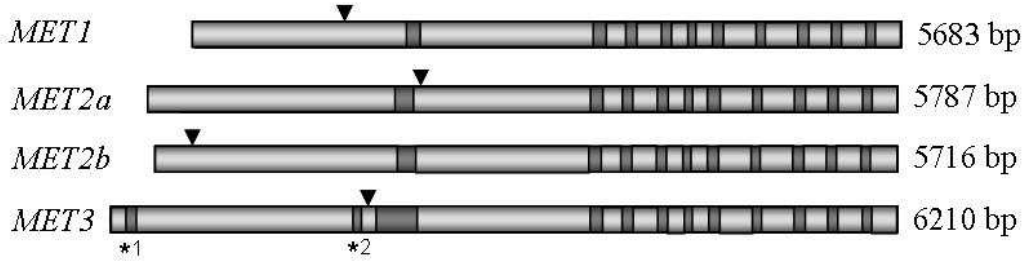


Figure 3.3 Schematic showing annotated intron positions in *MET1* family genes. Light grey regions represent exons and dark grey bands mark annotated intron positions. * Marks annotated introns unique to *MET3*. 1 marks the stop codon and 2 marks the base deletion discussed in accompanying text. Triangles mark the T-DNA insertion site positions discussed in **3.3.3.1**.

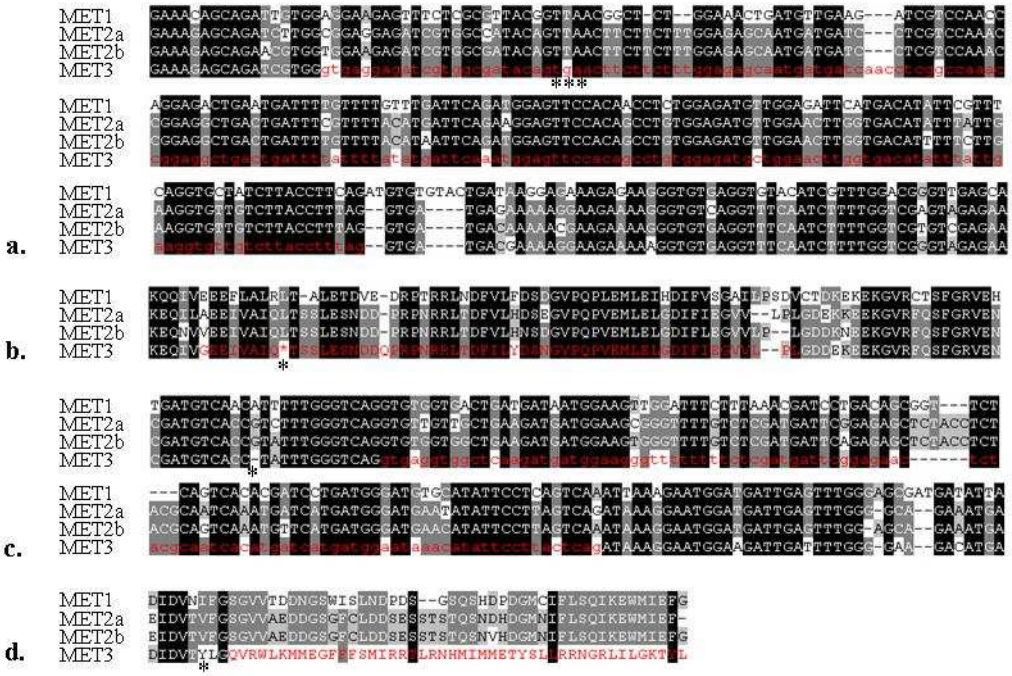


Figure 3.4 DNA and amino acid sequence alignments of *MET1* family genes spanning *MET3* annotated introns one (a. and b.) and two (c. and d.). a. and **c.** Alignments of the coding regions of *MET1*, *MET2a* and *MET2b* with the genomic sequence of MET3. Annotated *MET3* introns are in lower case letters and are highlighted in red. **b.** and **d.** Alignments of the amino acid sequences of MET1, MET2a and MET2b with that of MET3 translated from a *MET3* coding region from which annotated *MET3* introns one and two have not been spliced out. The sequence encoded by annotated *MET3* introns is highlighted in red. * marks the positions of the *MET3* one stop codon mutation (**a**. and **b.**) and the *MET3* base deletion (**c.** and **d.**).

These findings raised the possibility that MET3 introns one and two may be incorrectly annotated. If *MET3* introns one and two are correctly annotated, then *MET3* encodes a full length DNA methyltransferase with a fragment deletion in the putative RFTS which is likely to inhibit DNA methyltransferase activity. However, if *MET3* introns one and two are incorrectly annotated, then *MET3* encodes a truncated protein lacking a methyltransferase domain.

To assess whether or not *MET3* introns one and two are correctly annotated, the *MET3* genomic sequence was analyzed using NetPlantGene, an *Arabidopsis* splice site prediction program (Hebsgaard, S. M. et al. 1996). NetPlantGene predicted neither a donor nor acceptor site for either intron one or two. This supports the suggestion that these *MET3* introns are incorrectly annotated and therefore *MET3* encodes a truncated protein in the *Arabidopsis* accession Col-0.

3.2.1.4 *Analysis of the expression profile of* MET1 *family genes*

The expression profile of each *MET1* family gene was analysed using Affymetric Genechip microarray data from the AtGenExpress developmental series for *Arabidopsis* Col-0, with the aim of uncovering clues to gene function (Schmid, M. et al. 2005). As there is no control against variation in the level of expression detected in different arrays, cautions should be taken in comparing expression levels between arrays. Nevertheless such comparisons can provide a useful overview of a gene expression profile.

In most tissues analysed, *MET1* transcript was detected at a high level and annotated present, *MET3* transcript was detected at a very low level but annotated present and *MET2a*/*MET2b* transcript, indistinguishable due to the probe set used, was detected at a very low level and annotated absent. Data from a selection of arrays is summarised in **Figure 3.5**. Interestingly, *MET2a*/*MET2b* transcript is annotated present in all array biological replicates only during the early stages of seed development, from mid globular/early heart embryo to mid/late torpedo embryo (**Figure 3.5c**). This suggests that *MET2a* and/or *MET2b* may be expressed tissue-specifically.

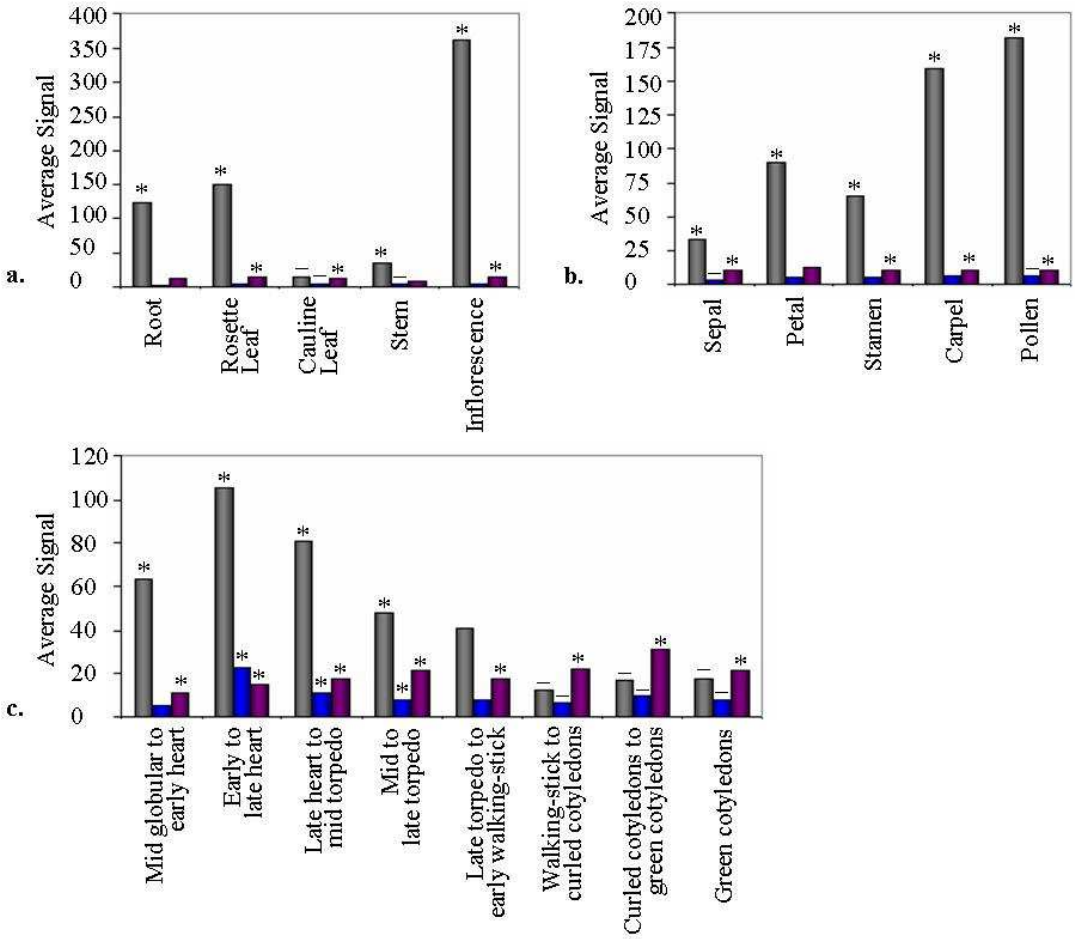


Figure 3.5 A summary of *MET1* family gene expression from microarray data. The average signal is the mean from three biological replicates. * and – mark when transcripts are called present or absent respectively, in all array biological replicates. **a.** Array data from various stages during plant development. **b.** Array data from stage 12 floral organs. **c.** Array data throughout seed development. Array sample IDs are shown below in brackets: root (ATGE_3), Rosette leaf (ATGE_17), Cauline leaf (ATGE_26), Stem (ATGE_27), Inflorescence (ATGE_29), Sepal (ATGE_34), Petal (ATGE_35), Stamen (ATGE_36), Carpel (ATGE_37), Pollen (ATGE_73), Mid globular to early heart (ATGE_76), Early to late heart (ATGE_77), Late heart to mid torpedo (ATGE_78), Mid to late torpedo (ATGE_79), Late torpedo to early walking-stick(ATGE_81), Walking-stick to curled cotyledons (ATGE_82), Curled cotyledons to green cotyledons (ATGE_83).

3.2.2 *Expression analysis of* MET2a, MET2b *and* MET3

To validate the expression profile of *MET1* family genes indicated from AtGenExpress microarray data, the expression profile of *MET2a*, *MET2b* and *MET3* was investigated using RT PCR.

3.2.2.1 MET2a *and* MET2b *expression*

AtGenExpress microarray data indicates that *MET2a* and/or *MET2b* may be expressed tissue-specifically during the early stages of seed development in the *Arabidopsis* accession Col-0 (**3.3.1.4**). In contrast, Genger et al. (1999) detected *MET2a* and/or *MET2b* transcript in a variety of vegetative and floral tissues of the accession C24 using RT PCR. In both analyses *MET2a* and *MET2b* transcripts were indistinguishable due to the probe/primer set design. To clarify the expression profile of these genes, RT PCR was used to compare *MET2a*/*MET2b* expression in leaf and 5DAP silique (heart stage) tissue from accessions Col-0 and C24. Primers and PCR conditions used were as described in Genger et al. (1999). In agreement with aforementioned analyses, *MET2a*/*MET2b* transcript was detected in silique tissue from both accessions and in leaf tissue from the C24 accession only (**Figure 3.6**). This indicates that the expression profile of these genes may be accession-specific.

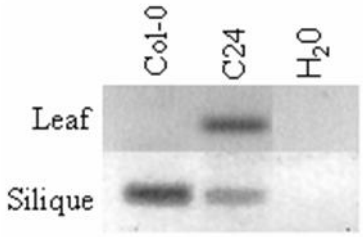


Figure 3.6 RT PCR comparing the expression of *MET2a*/*MET2b* in the *Arabidopsis* accessions Col-0 and C24.

To investigate whether one or both *MET2* genes are expressed, the RT PCR product amplified from Col-0 silique was sequenced. Sequence reads show polymorphisms between the *MET2a* and *MET2b* genes indicating that transcripts from both genes were amplified by RT PCR (**Figure 3.7**). This provides the first reported verification that both *MET2a* and *MET2b* are expressed.

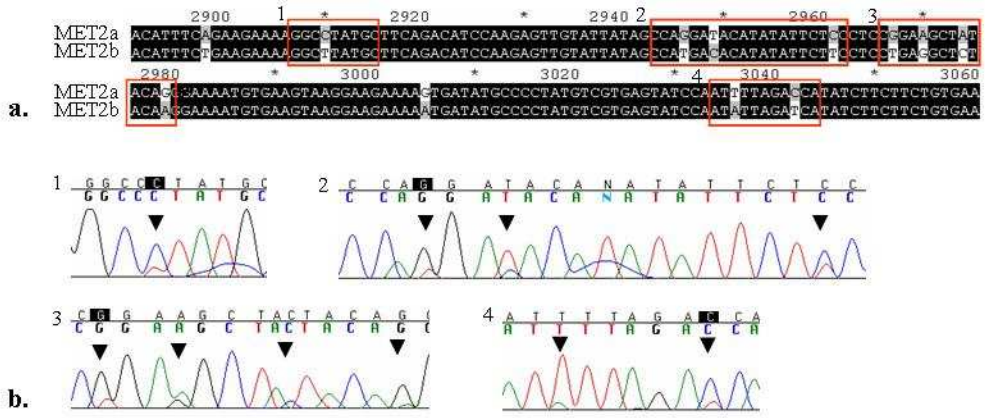


Figure 3.7 *MET2a* and *MET2b* polymorphisms identified in sequenced RT PCR fragment. a. An alignment of the *MET2a* and *MET2b* region amplified by the RT PCR. Red boxes mark 4 regions of polymorphism. **b.** Sequencing reads of the boxed regions marked in **a**. Arrow heads mark polymorphisms.

3.2.2.2 MET3 *expression*

In silico analysis of the Col-0 *MET3* sequence indicated that *MET3* introns one and two may be incorrectly annotated. Consequently, the coding region of Col-0 *MET3* may be disrupted by a stop codon and a nucleotide deletion and therefore encode a truncated protein lacking the methyltransferase domain (**3.3.1.3**).

To further investigate whether or not *MET3* introns one and two are correctly annotated, attempts were made to amplify a region spanning the first three *MET3* introns from 5DAP silique cDNA from the accessions Col-0 and C24. No fragment was amplified from Col-0, but one was successfully amplified from C24. Sequencing of this revealed that introns one and two are *not* spliced from the C24 *MET3* transcript (**Figure 3.8**). This finding is consistent with the *in silico* analysis and provides further evidence that MET3 introns have been incorrectly annotated.

Interestingly, polymorphisms between the sequenced region of C24 *MET3 cDNA* and the corresponding region of Col-0 *MET3,* reveal that C24 *MET3* has neither the stop codon nor the deletion that apparently disrupt the encoding region of Col-0 *MET3* (**Figure 3.8**). It is therefore possible that C24 *MET3* encodes a functional DNA methyltransferase despite the evidence suggesting that Col-0 *MET3* encodes a truncated protein. However, it is possible that another mutation downstream of the sequenced region disrupts C24 *MET*3 function.

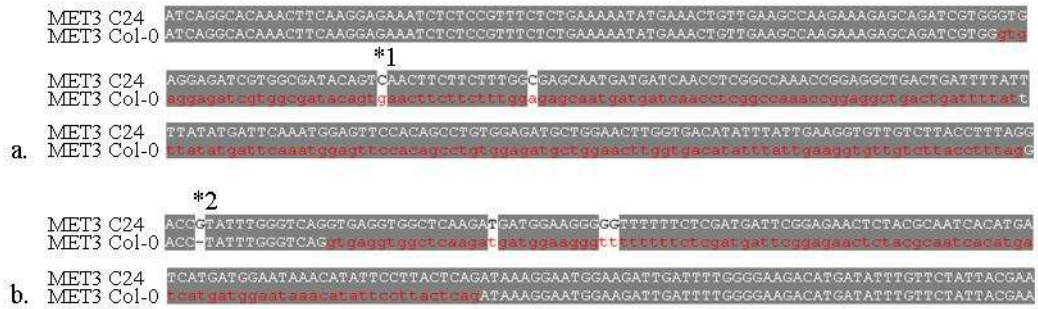


Figure 3.8 Alignments of sequenced C24 *MET3* cDNA and Col-0 *MET3* genomic DNA spanning annotated *MET3* introns one (a) and two (b). Annotated Col-0 *MET3* introns are in lower case letters and are highlighted in red. ∗1 marks a polymorphism converting the stop codon of Col-0 *MET3* (TGA) to serine (TCA) in C24 *MET3*. ∗2 marks the absence of a base deletion in C24 *MET3*.

3.2.3 MET1 *family T-DNA insertion lines*

A reverse genetics approach was used to further investigate the function of *MET1* family genes. A T-DNA insertion line for each *MET1* family gene was selected from the SALK database at NASC by Sushma Tiwari (University of Bath). *MET1*, *MET2a*, *MET2b* and *MET3* T-DNA insertion lines have been named *met1-9*, *met2a-1*, *met2b-1* and *met3-1* respectively. Sushma Tiwari also designed primers to genotype plants with respect to each *MET1* family gene T-DNA insertion using PCR. Homozygous plants for each *MET1* family T-DNA insertion lines were selected and as the first generation of homozygotes these are referred to as G1[*]. *met2a-1*, *met2b-1* and *met3-1* homozygous lines were maintained through successive generations of inbreeding (G2, G3 etc.). However, due to their reduced fertility, homozygotes *met1-9* plants were always generated from the self-fertilized heterozygotes and hence were always G1. Further reference to *met1-9*, *met2a-1*, *met2b-1* and *met3-1* lines refers to homozygous plants unless otherwise stated.

3.2.3.1 *T-DNA insertion sites*

The site at which T-DNA is inserted into a gene can indicate the likelihood that gene function is knocked-out. The SALK database provides an approximate insertion site for each T-DNA. To locate the T-DNA insertion site in *met1-9*, *met2a-1*, *met2b-1* and *met3-1* lines more precisely, PCR was used to amplify a region from the left border of the T-DNA into each respective *MET1* family gene. PCR fragments were then sequenced and the

[*] T-DNA insertion lines obtained from the SALK database are segregating T3 lines and therefore G1 homozygotes are likely to be T4 lines.

sequence reads were aligned with the genomic sequence of the relevant *MET1* family gene and the T-DNA lift border sequence. For all four *MET1* family T-DNA insertion lines, the T-DNA insertion site was located to within a few base pairs* (**Figure 3.9**) and found to be within an intronic sequence that is 5' to the DNA methyltransferase catalytic domain (**Figure 3.3** shows the T-DNA insertion sites in relation to exon/intron boundaries). This indicates that all four lines are likely to be null mutants with respect to DNA methyltransferase activity. However, this needs to be confirmed by expression analysis.

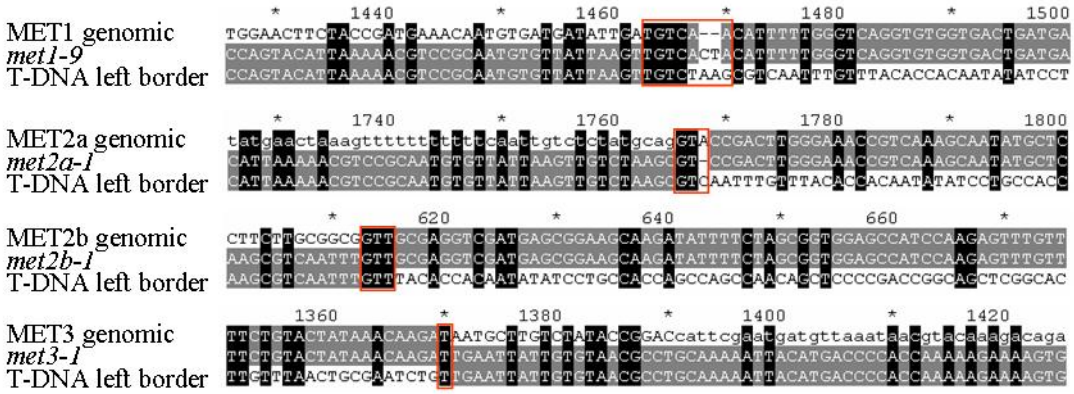


Figure 3.9 *met1-9, met2a-1*, *met2b-1*and *met3-1* T-DNA insertion sites. Red blocks mark the T-DNA insertion region for each gene. In all cases sequencing revealed that part of the T-DNA left border sequence was lost or scrambled; this is a common occurrence during transformation.

3.2.4 *Methylation analysis of* MET1 *family T-DNA insertion lines*

To assess the putative methyltransferase activity of MET2a, MET2b and MET3, *met2a-1*, *met2b-1* and *met3-1* lines were tested for hypomethylation of the180bp centromeric repeat sequence and the *FWA* loci.

3.2.4.1 *Methylation of the 180 bp centromeric repeat sequence*

The 180bp centromeric repeat is highly methylated in WT plants (Martinez-Zapater, J. M. et al. 1986). Southern hybridisation was used to test for hypomethylation of this repeat in each *MET1* family T-DNA insertion line. Leaf DNA from each line was digested with *Hpa*II or *Msp*I and probed with the 180 centromeric repeat sequence. Both enzymes cleave the CCGG sequence but cleavage by *Hap*II is inhibited by cytosine methylation (McClelland, M. et al. 1994). WT DNA remained largely undigested by *HpaII* indicating

* To narrow down the insertion site region further and determine whether any deletions or rearrangement of the donor gene has occurred, it is necessary to also sequence from the right border.

CCGG sites were methylated, whereas DNA from *met1-9* homozygous and heterozygous lines was equally digested by *HapII* and *MspI* indicating that these lines are hypomethylated at these sites (**Figure 3.10**). DNA from *met2a-1*, *met2b-1* and *met3-1* lines had a similar digestion pattern to WT DNA suggesting that these lines are methylated at CCGG sites. These results confirms that *MET1* is required to maintain methylation at CCGG sites but suggest that *MET2a*, *MET2*b and *MET3* do not play a role in maintaining methylation at these sites, at least in leaf tissue.

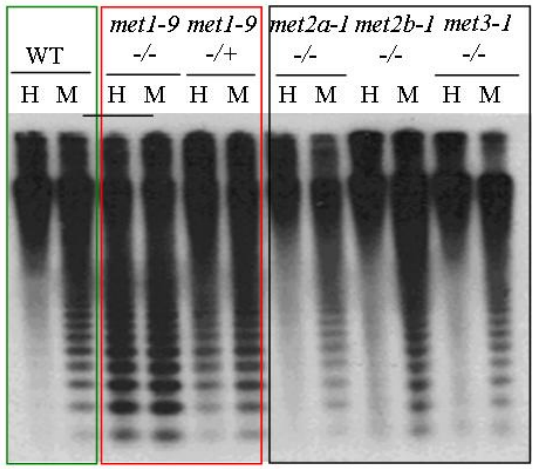


Figure 3.10 Southern hybridisation analysing CG methylation at the 180 bp centromeric repeat sequence from *MET1* family T-DNA insertion lines. DNA digested with methylation sensitive *Hpa*II (H) and M methylation insensitive MspI (H). -/+ indicates that lines were heterozygous with respect to the T-DNA insertion and -/- indicates that lines were homozygous.

3.2.4.2 *Methylation of the* FWA *locus*

Silencing of *FWA* is dependent on the methylation of two direct repeat sequences 5' to the *FWA* translational start (Jullien, P. E. et al. 2006b, Kinoshita, Y. et al. 2007, Soppe, W. J. J. et al. 2000). To further test for hypomethylation in *met2a-1*, *met2a-1* and *met3-1* lines, RT PCR was used to test for ectopic expression of *FWA* in leaf tissue from these lines. Ectopic *FWA* expression was not detected in *met2a-1*, *met2a-1* and *met3-1* lines but was evident in the *met1-9* line (**Figure 3.11**). This indicates that *MET1* is required for the vegetative silencing of *FWA* and suggests that neither *MET2a* nor *MET2b* or *MET3* play a role in maintaining the silencing of this locus.

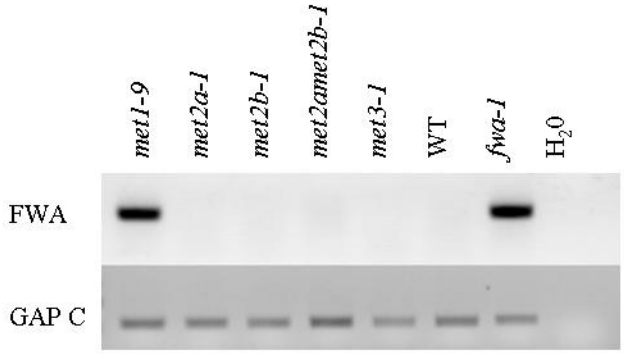


Figure 3.11 RT PCR analysis of *FWA* expression in leaf tissue from *MET1* family T-DNA insertion lines. A fragment of the *GAPC* gene was amplified as a control.

3.2.5 *Phenotype analysis of* MET1 *family T-DNA insertion lines*

Mutants that are hypomethylated at CG sites display distinct phenotype abnormalities (Finnegan, E. J. et al. 1996, Kakutani, T. et al. 1995, Ronemus, M. J. et al. 1996). To further test for hypomethylation in *met2a-1*, *met2b-1* and *met3-1* lines, the phenotype of these lines was analysed.

3.2.5.1 *The effect of inbreeding on the phenotype of* met2a-1, met2b-1 *and* met3-1 *lines*

Preliminary analysis revealed that the phenotype of *met2a-1*, *met2b-1* and *met3-1* G1 lines was comparable to WT plants. Some hypomethylated lines, including the *decreased dna methylation* (*ddm1*) mutant and *MET1*as lines, become increasingly hypomethylated and display increasingly severe developmental abnormalities after successive generations of inbreeding (Finnegan, E. J. et al. 1996, Kakutani, T. et al. 1996). In an attempt to expose any phenotype abnormalities caused by loss of MET2a, MET2b or MET3 function, *met2a-1*, *met2b-1* and *met3-1* lines were inbred for 8 generations and phenotype characteristics that are known to be altered by hypomethylation were analysed in G2, G4 and G9 *met2a-1*, *met2b-1* and *met3-1* lines.

No phenotype variations correlating with the level of inbreeding were detected in *met2a-1, met2b-1 or met3-1* lines (**Table 3.1**), indicating that loss of *MET2a*, *MET2b* and *MET3* activity for successive generations does not result in the accumulation of developmental defects. The low of level of variation detected between lines of the same genotype that were inbreed for a different number of generations, is likely to be caused by subtle differences in growth conditions, for example position in the growth room or level of watering. A larger data set could reduce this variation.

Table 3.1 Phenotype analysis of MET1 family T-DNA insertion lines

Lines	Flowering Time [a]	Leaf Area (mm^2) [b]	Height (cm) [c]	Branching [d]	Floral Abnormalities [e]	Seed Weight (μg) [f]
met1-9 G1	32.9 ± 1.0 34	2.7 ± 0.14 2.7	20.9 ± 1.8 21.0	15.9 ± 1.1 17	54 ± 8.5 50	12.8 ± 0.4 12.9
met2a-1 G2	15.5 ± 0.2 16	7.9 ± 0.4 8.1	30.4 ± 1.8 33.5	3.0 ± 0.2 3	28 ± 4.2 30	20.3 ± 0.8 21.0
met2a-1 G4	15.1± 0.3 15	6.1 ± 0.3 6.2	36.1 ± 1.6 41.4	3.1±0.1 3	18 ± 3.6 20	18.1 ± 0.6 17.5
met2a-1 G9	16.4 ± 0.3 16	6.1 ± 0.2 6.2	34.4±1.4 33.8	3.4 ± 0.2 3	21 ± 4.3 20	22.1± 0.1 22.1
met2b-1 G2	15.1± 0.3 15.5	7.9 ± 0.3 7.8	33.1 ± 1.8 35.0	3.1±0.2 3	23 ± 4.0 20	19.9 ± 1.1 21.0
met2b-1 G4	15.0 ± 0.3 15	5.8 ± 0.3 5.7	28.7 ± 2.5 32.0	3.1±0.2 3	20 ± 4.7 20	20.1± 0.9 19.8
met2b-1 G9	17.2 ± 0.3 17	6.8 ± 0.1 6.9	35.7 ± 1.4 37.5	3.7 ± 0.2 4	22 ± 5.7 15	21.3 ± 0.2 21.5
met2amet2b-1 G2	16.4 ± 0.4 16	5.4 ± 0.3 5.4	35.0 ± 1.4 36.30	3 ± 0.2 3	27 ± 3.0 30	22.4 ± 0.9 22.1
met3-1 G2	15.5 ± 0.3 16	7.1 ± 0.3 6.9	32.3 ± 2.1 37.9	3.1 ± 0.2 3	30 ± 3.9 30	19.1 ± 0.3 18.8
met3-1 G4	15.7 ± 0.3 16	7.5 ± 0.3 7.5	33.6 ± 1.1 35.6	4.6 ± 0.3 5	23 ± 4.2 25	21.1 ± 0.7 20.
met3-1 G9	14.7 ± 0.3 15	6.5 ± 0.5 6.6	31.8 ± 1.1 31.7	3.4 ± 0.2 3	24 ± 3.7 25	20.9 ± 0.4 20.7
WT	15.9 ± 0.4 16	7.5 ± 0.3 7.8	37.9 ± 1.2 37.3	3±0.9 3	33±5.6 30	20.1 ± 0.5 19.8
P^1	0.000♣	0.000♣	0.002♣	0.001♣	0.015♣	0.000♣♣
P^2	0.000 ♣	0.000 ♣♣	0.001 ♣	0.000 ♣	0.014♣	0.001♣♣

Mean ± SEM and median are shown. n=9 or 10 (for seed weight n=3).
[a] Flowering-time measured as number of rosettes leaves produced before bolting
[b] Area of largest rosette leaf measured two weeks after bolting
[c] Height of primary inflorescence measured two weeks after bolting
[d] Number of branches from primary inflorescence measured two weeks after bolting
[e] Percentage of flowers with abnormal numbers of floral organs or homeotic mutations
[f] Seed weight calculated from the average seed weight per pod of five pods on three plants per line
P^1 shows the P value obtained from a Mood's Median test (♣) or ANOVA (♣♣) comparing data a phenotype characteristic of all lines shaded.
P^2 shows the P value obtained from a Mann-Whitney U test (♣) or T-test (♣♣) comparing the line that has the most similar phenotype to *met1-9,*with *met1-9*.

3.2.5.2 *Phenotype comparison of* MET1 *family T-DNA insertion lines*

To further test whether or not loss *MET2a*, *MET2b* and *MET3* function causes phenotype abnormalities associated with hypomethylation, the phenotype of G9 *met2a-1, met2b-1* and *met3-1* lines was compared with that of *met1-9* and WT lines. **Table 3.1** and **Figure 3.12** show that the phenotype of *met2a-1, met2b-1 and met3-1* lines closely resembled that WT plants, whereas the *met1-9* line was significantly different. The *met1-9* line was late flowering and produced twice as many rosette leaves as the other lines. This is consistent with the report of ectopic *FWA* expression in the *met1-9* line (**3.2.4.2**), as ectopic *FWA*

expression reportedly causes delayed flowering (Soppe, W. J. J. et al. 2000). Leaves from the *met1-9* plants were typically darker, more jagged and smaller than those from the other lines; the area of the largest *met1-9* leaf was less than half of that from the other lines.

At two weeks after bolting the *met1-9* line was approximately two-thirds the height of the other lines but and had around five times more auxiliary flowering shoots. The *met1-9* line also had twice as many floral mutations as other lines; these commonly included an increased number of anthers and unfused carpels. Fertility varied greatly between individual *met1-9* plants but commonly plants set very few seeds per pod, moreover the seed produced from these plants weighed nearly 50% less than that from the other lines. In contract, *met2a-1, met2b-1, met3-1* lines were fully fertile and produced seed with a comparable weight to WT.

The phenotype of the *met1-9* line supports a role for MET1 in maintaining methylation to regulate gene expression throughout development. Conversely, the phenotype of *met2a-1, met2b-1* and *met3-1* plants provides further evidence to suggest that these lines are not hypomethylation and indicates that MET2a, MET2b and MET3 do not play a role in regulating development.

Figure 3.12 *MET1* family T-DNA insertion lines at two weeks after bolting.
Scale bar, 2 cm

3.2.6 *Imprinting analysis of* **MET1** *family T-DNA insertion lines*

It was hypothesised that MET2a, MET2b and MET3 play a role in controlling imprinted gene expression either redundantly or complementarily to MET1 and possibly in a tissue- or locus-specific manor (**1.4.2.3**). To test this hypothesis, three approaches were used to assess imprinting in each *MET1* family T-DNA insertion line.

3.2.6.1 *Parent-of-origin-specific effects of loss of* **MET1** *family gene expression on seed weight*

Inheritance of hypomethylated genomes from *MET1*as lines has a parent-of-origin-specific effect on seed development; [WT X *MET1*as] seed has a low seed weight, whereas [*MET1*as X WT] seed has a high seed weight (Adams, S. et al. 2000). This is consistent with the model that DNA methylation is required to silence maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes (Adams, S. et al. 2000). To determine which MET1 family members play a role in controlling the imprinting of these genes, the weight of seed from reciprocal crosses between *met1-9*, *met2a-1, met2b-1* and *met3-1* lines and WT plants was analysed (**Figure 3.13**).

The weight of [*met1-9* X WT] seed was significantly different to [WT x *met1-9*] seed (t=-17.91, d.f. =14, P=0.000); [*met1-9* X WT] seed was approximately 60 % heavier than WT seed, whereas [WT X *met1-9*] seed was 35 % lighter. This is consistent with the trend reported for reciprocal crosses between the *MET1*as line and WT plants (Adams, S. et al. 2000), and supports the suggestion that MET1 plays a fundamental role in silencing maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes. However, fewer seeds per pod were produced from a *met1-9* X WT cross compared with the reciprocal cross and in *Arabidopsis,* seed weight is positively correlated with reduced seed set (Adams, S. 2002). It is therefore possible that the high weight of [*met1-9* X WT] seed is the result of low seed set. To investigate this possibility, a Pearson correlation was performed to test for a correlation between seed weight and the number of seed set per pod from reciprocal *met1-9* x WT crosses. No significant correlation was detected between seed weight and the number of seed per pod from either the *met1-9* X WT or WT X met1-9 cross (P= 0.342 and P=0.919 respectively). This indicates that the heavy weight of [*met1-9* X WT] seed is unlikely to be a consequence of reduced seed set *per se* and supports the model that maternal hypomethylation results in the released silenced maternally-inherited endosperm-promoting genes.

Reduced seed set from the *met1-9* X WT crosses it likely to be caused by a sporophyte of hypomethylation on the seed parent. This is consistent with floral mutations reported for met1-9 plants (see section **3.2.5** and **Table 3.1**) and with reports that hypomethylation deregulates the expression of the floral developmental regulators *SUPERMAN* and *AGAMUS* (Jacobsen, S. E. and Meyerowitz, E. M. 1997 and Jacobson, S. E. et al. 2000). Reduced seed set from *met1-9* plants selfed was also commonly observed (data not shown) but was not correlated with increased seed weight (see section **3.2.5** and **Table 3.1**). This indicates that the increased weight of [*met1-9* X WT] seed is unlikely to result from maternal sporophytic effects of hypomethylation *per se* but instead provides further support for the model that maternal hypomethylation results in the released silenced maternally-inherited endosperm-promoting genes.

In contrast to the complementary seed weights produced from reciprocal *met1-9* X WT crosses, no significant difference was detected between the seed weights from reciprocal crosses between *met2a-1*, *met2b-1* and *met3-1* lines and WT plants ((T=-1.76, D.F.=28, P=0.11), (t=1.32, d.f.=22, P=0.20), (t=-0.49, d.f.=18, P=0.63) respectively). These results provide no evidence that MET2a, MET2b and MET3 are required for the imprinting of genes controlling seed size and therefore indicate that these putative DNA methyltransferases play no role in the imprinting of these loci.

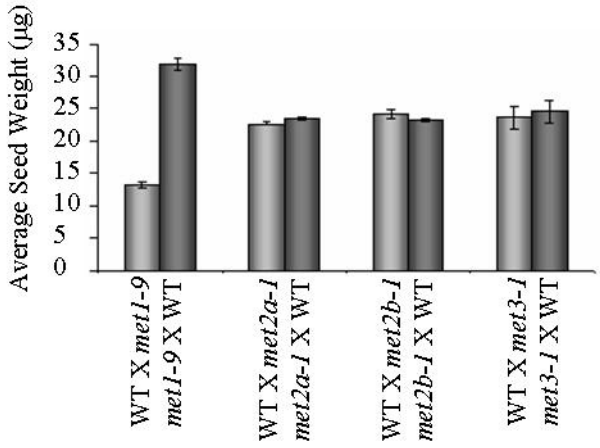


Figure 3.13 Seed weights from reciprocal crosses between *MET1* family T-DNA insertion lines and WT plants. Average seed weight calculated as the mean of the average weight of seed from five pods on three plants per cross. Error bars show standard error.

3.2.6.2 *Paternal effects of loss of* MET1 *family gene expression on seed abortion of* mea *mutants*

A potential role for MET2a, MET2b and MET3 in maintaining the silencing of paternally-inherited endosperm-inhibiting genes was further tested by assessing the ability to pollen from *MET1* family T-DNA insertion lines to rescue seed abortion of *mea* mutants. Seed abortion of maternal *mea* mutants is correlated with endosperm over-proliferation and is rescued by hypomethylation pollen (Grossniklaus, U. et al. 1998, Kiyosue, T. et al. 1999, Vielle-Calzada, J. P. et al. 1999). This rescue is suggested to result from the released silencing of endosperm-inhibiting genes inherited from the hypomethylated pollen (Spielman, M. et al. 2001). This is consistent with the model suggesting DNA methylation maintains the silencing of paternally-inherited endosperm-inhibiting genes (Adams, S. et al. 2000). The ability of pollen from *MET1* family T-DNA insertion lines to rescue *mea* seed abortion would therefore indicate that imprinting of endosperm-inhibiting genes is disrupted in these lines and would thus indicate which MET1 family members are required for this imprinting

mea mutants were emasculated and pollinated by homozygous plants from each *MET1* family T-DNA insertion line. At 14DAP the number of aborted vs. viable seeds per pod from each cross was scored (shrivelled brown seed was scored aborted and plum green seed was scored viable) (**Table 3.2**). When *mea* mutants were self-fertilized 97% of seed aborted, whereas when *mea* mutants were pollinated by the *met1-9* line only 3% of seed aborted. In contrast, when pollinated by the *met2a-1*, *met2b-1* or *met3-1* lines the level of aborted was comparable to that observed *mea* mutants self fertilized. These results are consistent with the suggestion that MET1 is required to silence paternally-inherited endosperm-inhibiting genes and provide no evidence that MET2a, MET2b and MET3 play a role in regulating the imprinting of these genes. However, it should be noted that the *mea* mutant is in a La-*er* background whereas the MET1 family T-DNA insertion lines are in a Col-0 background. The La-er/Col-0 hybrid background created from crosses between *mea* and MET1 family T-DNA insertion lines could affect the rescue of seed abortion. To overcome this problem, the crosses should be repeated using a *mea* mutant and T-DNA insertion lines in the same accession background.

Table 3.2 Percentage seed aborted from crosses between *mea* mutants and *MET1* family T-DNA insertion lines

Cross [a]	Mean % Seed Abortion [b]	SEM [c]	Total number of seeds analyzed
mea (La-*er*) X *met1-9* (Col-0)	3.5	1.6	1866
mea (La-*er*) X *met2a-1* (Col-0)	99.5	0.5	654
mea (La-*er*) X *met2b-1* (Col-0)	98.6	0.9	243
mea (La-*er*) X *met2amet2b-1* (Col-0)	97.2	0.6	378
mea (La-*er*) X *met3* (Col-0)	97.9	1.2	319
mea (La-*er*) X *mea* (La-*er*)	97.0	0.7	1305

[a] Genetic background of plants shown in brackets
[b] Mean of the average percentage rescue of three/five pods from three mea mutant plants
[c] SEM seed abortion from three *mea* mutant plants

3.2.6.3 *The effect of loss of* MET1 *family gene expression on* FWA *imprinting*

FWA is imprinted and normally maternally-expressed/paternally-silenced during endosperm development (Kinoshita, T. et al. 2004). However, when paternally-inherited from a *met1* mutant background, *FWA* is ectopically expressed indicating that *FWA* imprinting is dependent on maintenance DNA methyltransferase activity (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). To test whether or not *MET2a*, *MET2b* and *MET3* participate in controlling the monoallelic expression of this locus, an *FWA*::*GFP* transcriptional reporter (Kinoshita, T. et al. 2004) was used to assess the parent-of-origin-specific expression of *FWA* inherited from *met2a-1*, *met2b-1* and *met3-1* backgrounds. It is likely that FWA constitutes a unique class of imprinted genes because, in contrast to the prediction that imprinted gene act to regulate endosperm proliferation (Haig, D. and Westoby, M. 1989, Haig, D. and Westoby, M. 1991), FWA appears to have no function within the seed. This test therefore complements the two described above.

Homozygous T-DNA insertion lines were crossed with plants homozygous for the reporter. PCR-based genotyping was then used to identify homozygous T-DNA insertion lines in the F2 generation and florescence microscopy was used to identify those which also inherited the *FWA*::*GFP* reporter[*]. These selected plants were reciprocally crossed with WT plants and 3DAP seed was analysed for *GFP* expression using florescence and confocal microscopy. In control crosses, lines carrying the *FWA*::*GFP* reporter in a WT background (WT *FWA*::*GFP*) were reciprocally crossed with WT plants and again 3DAP seed was analysed for reporter expression by florescence microscopy.

[*] Strong *GFP* expression was observed in the endosperm of 3DAP seed from plants carrying the *FWA*::*GFP* reporter.

GFP was present in [WT *FWA*::*GFP* X WT] seed but not [WT X WT *FWA*::*GFP*] seed indicating that the reporter is imprinted similarly to *FWA* when inherited from a WT background (Kinoshita, T. et al. 2004) (**Figure 3.14a**). When inherited from a *met1-9* background, imprinting was disrupted and GFP was present in both [*met1-9 FWA*::*GFP* X WT] and [WT x *met1-9*, *FWA*::*GFP*] seed (**Figure 3.14b**). In contrast, when the reporter was inherited from a *met2a-1*, *met2b-1* or *met3-1* background, imprinting of the reporter was maintained (~450 seeds viewed per cross). This indicates that MET1 is required to maintain *FWA* imprinting whereas MET2a, MET2b and MET3 are dispensable for this function.

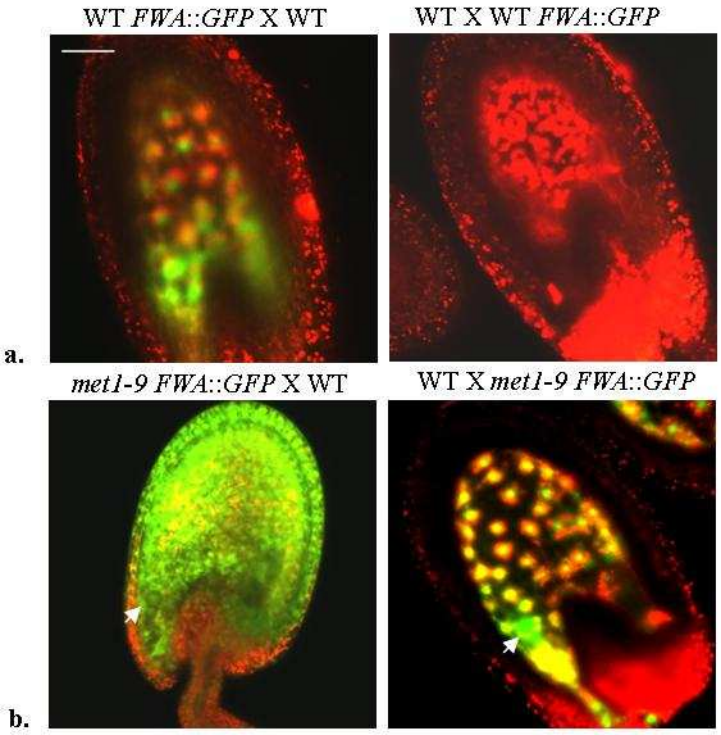


Figure 3.14 Expression of the *FWA*::*GFP* reporter in when maternally (left) or paternally (right) inherited from a WT (a) and *met1-9* (b) background. White arrows points to the embryo. Scale bar, 50 μm.

3.2.7 met2a-1met2b-1 *double mutants*

The results discussed above indicate that *MET2a* and *MET2b* encode functional DNA methyltransferases yet single *met2a-1* and *met2b-1* loss-of-function mutants were not detectably hypomethylated and did not exhibit any phenotype abnormalities or altered imprinting behaviour. This raised the possibility that these genes act redundantly. To test

for redundancy a *met2a-1met2b-1* double mutant was made and analysed for hypomethylation and altered imprinting.

3.2.7.1 *Methylation analysis of the* met2a-1met2b-1 *line*

To analyse methylation in the *met2a-1met2b-1* line, RT PCR was used to test for ectopic expression of *FWA* in leaf tissue from this line as described in **3.2.4.1**. Ectopic *FWA* expression was not detected (Refer back to **Figure 3.11**) indicating that the *FWA* locus was not hypomethylated and MET2a and MET2b do not act redundantly to maintain the silencing of this locus.

3.2.7.2 *Phenotype analysis of the* met2a-1met2b-1 *line*

To further test for hypomethylation, the phenotype of the G2 *met2a-1met2b-1* plants was assessed for abnormalities. These plants were indistinguishable from WT and did not display any characteristics diagnostic of hypomethylation (Ref back to **Table 3.1**). Consistent with the methylation analysis described above, this indicates that *met2a-1met2b-1* plants are not hypomethylated at CG sites and MET2a and MET2b do not act redundantly to maintain CG methylation.

3.2.7.3 *Imprinting analysis of the* met2a-1met2b-1 *line*

To test whether MET2a and MET2b act redundantly to control imprinted gene expression, the *met2a-1met2b-1* line was subjected to the three imprinting tests described in **3.3.6**.

Firstly, seed from reciprocal crosses between the *met2a-1met2b-1* and WT was analysed. In contrast to the crosses described previously (**3.3.6.1**), plants were subjected to restricted seed set as this is believed to provide a more accurate comparison of seed weight. No significant difference was detected between the weight of [*met2a-1met2b-1* X WT] and [WT X *met2a-1met2b-1*] seed (W=20.0, N_1=5, N_2=5, P=0.143) indicating that MET2a and MET2b do not act redundantly to silence maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes.

Secondly, to further test for MET2a and MET2b redundancy in silencing paternally-inherited endosperm-inhibiting genes, the ability of *met2a-1met2b-1* pollen to rescue *mea* seed abortion was assessed. The level of seed abortion from *mea* X *met2a-1met2b-1* crosses was comparable to that observed when *mea* mutants were self-fertilized (Refer

back to **Table 3.2**). This is consentient with the suggestion that MET2a and MET2b do not act redundant to silence paternally-inherited endosperm-inhibiting genes.

Thirdly, to test whether MET2a and MET2b act redundantly to control *FWA* imprinting, the *FWA*::*GFP* reporter was used to assess *FWA* expression in the endosperm of seed maternally and paternally-derived from *met2a-1met2b-1* plants. Homozygous *met2a-1met2b-1* plants carrying the *FWA*::*GFP* reporter were reciprocally crossed with WT plants and 3DAP seed was analysed for *GFP* expression. GFP was present in [*met2a-1met2b-1 FWA*::*GFP* X WT] seed but not [WT X *met2a-1met2b-1 FWA*::*GFP*] seed; imprinting of the reporter was therefore not disrupted. This finding is consistent findings from the previous two imprinting tests and indicates that MET2a and MET2b to not act redundantly to control imprinted gene expression.

3.3 *Discussion*

The aim of the work described in this chapter was to determine which *MET1* family genes play a role in controlling plant genomic imprinting. This section reviews the functionality of *MET2a*, *MET2b* and *MET3* and their role in imprinting. Additionally, this section briefly considers the role of DNA methyltransferases in imprinting in plants and mammals and why some plants have a *MET1* gene family.

3.3.1 *The functionality of* MET1 *family genes*

3.3.1.1 MET2a *and* MET2b

The putative amino acid sequence of MET2a and MET2b is highly similar to MET1 and incorporates all the putative functional domains identified in MET1. These include a NLS, a RFTS and BAH which indicate that MET2a and MET2b can enter the nucleus, target the replication fork (the site of maintenance DNA methyltransferase catalytic activity) and form protein-protein interactions with gene silencing machinery. Additionally, MET2a and MET2b have the eight conserved methyltransferase motifs that are fundamental for, and indicative of, DNA methyltransferase activity. Ka/Ks analysis, comparing the coding region of MET2a and MET2b with that of MET1, provides strong evidence of purifying selection at these putative functional domains suggesting that have been conserved, presumably to maintain their function. Together these findings provide strong evidence that *MET2a* and *MET2b* encode functional maintenance DNA methyltransferases.

Despite this, no evidence for hypomethylation was detected in *met2a-1, met2b-1* or *met2a-1met2b-1* lines. One explanation for this discrepancy is that the hypomethylation induced in these lines was not detectable by the approaches used here. In contrast to *MET1*, *MET2a* and *MET2b* may have a very restricted pattern of expression, either temporally, spatially or both, rendering hypomethylation effects difficult to detect. In agreement with this suggestion, AtGenExpress microarray data indicates that *MET2a* and/or *MET2b* transcript is detectable only during early seed development in the accession Col-0. Similarly, RT PCR analysed detected *MET2a* and *MET2b* transcript in silique tissue but not in leaf tissue from WT (Col-0) plants. Both finding strongly suggests that *MET2a* and *MET2b* do indeed have a highly restricted pattern of expression and therefore hypomethylation may only be induced tissue-specifically in loss-of-function lines.

Alternatively, hypomethylation in the loss-function lines would not be been detected if MET2a and MET2b methylate sequence motifs not analyzed by the approaches described. However, this assumes that MET2a/2b and MET1 maintain the methylation of different sequences and therefore must have distinct target recognition domains (TRD). In prokaryotic methyltransferases, a target recognition domain (TRD), which determines the DNA sequence that the enzymes methylate, is located between DNA methyltransferases motifs VII and IX (Klimasauskas, S. et al. 1991, Wilke, K. et al. 1988). However it is not know whether such a domain exists in plant methyltransferases.

If plant methyltransferases have a TRD between motifs VII and IX and if MET1 and MET2a/2b have diverged to methylate different sequences, it is expected that evidence of positive selection to alter amino acid sequence would be evident in this region. Conversely, if MET1 and MET2a/2b have evolved to methylate the same sequences, it is expected that evidence if stabilizing selection would be evident. However, Ka/Ks ratio analysis indicates that this region is subject to only weak stabilizing selection/neutral selection (Ka/Ks~1) (Refer back to **Figure 3.3**). The region between motifs VII and IX is therefore unlikely to constitute a TRD in plant DNA methyltransferases and it is possible that plant DNA methyltransferase do not possess such a domain.

In agreement with this suggestion, it is likely that the mammalian DNA methyltransferase DNMT1 does not have a TRD. Proteolytic removal of the N-terminal of DNMT1 destroys its ability to discriminate hemimethylated and unmethylated DNA, indicating that the C-

terminal region, including the catalytic motifs, does not direct DNMT1 to target sequences (Bester, T. H 1992). An N-terminal region of DNMT1 (amino acids 122-417) was shown to target hemimethylated CG sequences and was annotated a TRD (Araujo, F. D. et al. 2001). However, this region was subsequently shown to be dispensable for the preferential methylation of hemimethylated DNA (Vilkaitis, G. et al. 2005). These findings suggest that eukaryotic DNA methyltransferases do not have a definite TRD and targeting of hemimethylated CG sequences may be an inherent property of the enzymes. Accordingly, it is probable that all MET1 family members target hemimethylated CG sequences and therefore MET2a and MET2b are unlikely to act in a sequence specific manner.

The functionality of *MET2a* and *MET2b* could be further investigated by testing for tissue-specific and sequence-specific hypomethylation during seed development in *met2a* and *met2b* loss-of-function mutants. Additionally, the predicted homology between the functionality of *MET2a*/*MET2b* and *MET1* could be verified by testing whether constructs expressing *MET2a*/*MET2b* cDNA from the *MET1* promoter can rescue the phenotype of *met1* loss-of-function mutants. However, such work was beyond the scope of this project.

3.3.1.2 MET3

MET3 is reported to encode a truncated protein in the *Arabidopsis* accession Col-0 due to an in-frame stop signal at codon 65 and a deletion around codon 243 which results in a frame shift (Genger, R. K et al. 1999). However, TAIR annotate introns which could splice out these mutations. Sequencing of *MET3* cDNA from the *Arabidopsis* accession C24 revealed that annotated *MET3* introns one and two are in fact not spliced out from the *MET3* transcript in this accession. The genomic sequence spanning the splice sites of these introns is identical in Col-0 and C24 suggesting that annotated *MET3* introns one and two are not spliced from *MET3* transcript in either accession (**Figure 3.9**). It is reasonable therefore to assume that *MET3* introns have been incorrectly annotated and the stop and frame shift mutations render the MET3 inactive in Col-0.

Moreover, annotated MET3 intron two is located within a putative RFTS. If *MET3* introns were correctly annotated, *MET3* would encode a protein with a fragment deletion in this putative functional domain. In DNMT1, a deletion within this domain prevents targeting of the protein to the site of catalytic activity (Leonhardt, H. et al. 1992). Consequently, irrespective of whether or not *MET3* introns have been correctly annotated, *MET3* is likely

to be a pseudogene in Col-0. In agreement with this suggestion, AtGenExpress microarray data indicates that *MET3* transcript is present in broad range of tissues, yet no hypomethylation or phenotype abnormalities were apparent in the *met3-1* line, indicating that methylation and development is not disrupted is this loss-of-function line.

3.3.2 *The role of* MET1 *family genes in controlling genomic imprinting*

To determine which MET1 family members play a role in controlling imprinted gene expression, three approaches were used to test for altered imprinting in MET1 family loss-of-function lines. This strategy enabled the potential role of each *MET1* family gene to be assessed independently.

The first approach tested whether inheritance of genomes from each *MET1* family T-DNA insertion line had a parent-of-origin-specific effect on seed development. Inheritance of genomes from *met2a-1*, *met2b-1*, *met2a-1met2b-1* and *met1-3* lines had little effect on seed weight (**Figure 3.13**), whereas inheritance of genomes from *met1-9* lines had a significant parent-of-origin-specific effect on seed weight. The weight of [*met1-9* x WT] seed closely resembled that reported for [*MET1*as x WT] seed and the weight of [WT x *met1-9*] seed resembled [WT x *MET1*as] seed (Figure 3.13 & Adams, S. et al. 2000). A similar trend has also been reported for reciprocal crosses between the *met1-6* mutant and WT plants (Xiao, W. et al. 2006a). These finding are consistent with the model that *MET1* is required to maintain the silencing of maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting genes (Adams, S. et al. 2000). Conversely, the inability of parent-of-origin-specific expression of *MET2a*, *MET2b* and *MET3* to affect seed size suggests that these genes play no role in controlling the imprinting of genes which regulate endosperm proliferation.

The second approach tested whether or not the paternal inheritance of genomes from each *MET1* family T-DNA insertion line could rescue seed abortion of *mea* mutants. Pollination of *mea* mutants with *met2a-1*, *met2b-1*, *met2a-1met2b-1* and *met1-3* pollen had little effect on seed abortion, whereas *met1-9* pollen dramatically reduced seed abortion. Again these results are consistent with the model that *MET1* is required to silence paternally-inherited endosperm-inhibiting genes whereas *MET2a*, *MET2* and *MET3* have no involvement in this function.

The third approach investigated which *MET1* family genes control *FWA* imprinting, by testing whether or not silencing of the *FWA*::*GFP* reporter is lost when paternally-inherited from each *MET1*-family T-DNA insertion line. *FWA::GFP* silencing was maintained when paternally-inherited from *met2a-1*, *met2b-1*, *met2a-1met2b-1* and *met1-3* lines but lost when paternally-inherited from the *met1-9* line. This indicates that *MET1* is required to maintain paternal silencing of *FWA* and is therefore necessary for the imprinting of this locus. This is consistent with the finding that *FWA* is ectopically expressed in endosperm when paternally-inherited from other *met1* mutants (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). Additionally, these results demonstrate that *MET2a*, *MET2b* and *MET3* play no role in imprinting *FWA*.

The combination of these three approaches tested for altered expression of a broad spectrum of imprinted loci and should therefore provide a reliable test for altered imprinting in experimental lines. The results provide strong evidence that *MET1* is required to maintain parent-of-origin-specific the silencing of imprinted loci whereas *MET2a*, *MET2b* and *MET3* apparently play no role in imprinted. Imprinting model 1 and 2 (Chapter 1, **Figure 1.5** and **Figure 1.6**) can therefore be refined to show that *MET1* is the sole maintenance DNA methyltransferase required imprinting. Future work can now focus on investigating the function of *MET1* in imprinting.

These findings also indicate that any potential off-target effects on *MET2a*, *MET2b* or *MET3* expression caused by *MET1*as expression, do not contribute to the altered imprinting reported in *MET1*as lines (Adams, S. et al. 2000). Parent-of-origin-specific expression of *met1-9* homozygotes closely resembles those described for *MET1*as lines, confirming that the *MET1*as line is a useful tool for studying the role of *MET1* in imprinting.

Based on sequence conservation, *MET2a* and *MET2b* apparently encode functional maintenance DNA methyltransferase. It is possible that the inability of *MET2a* and/or *MET2b* to compensate for *MET1* loss-of-function is the consequence of their potential tissue-specific expression, and not their functionality. Proposed imprinting models 1 and 2 predict that maintenance DNA methyltransferase activity is required throughout sporophytic development to enable imprinting in the next generation (Chapter 1, section

1.4.2.6). The inability of tissue-specific *MET2a/MET2* expression to compensate for *MET1* loss-of-function agrees with this prediction.

3.3.3 *The role of DNA methyltransferases in genomic imprinting in mammals and plants*

The results described here enrich an interesting comparison of DNA methylation-dependent imprinting in mammals and plants. In mammals, imprinting appears to be achieved by the sex-specific methylation of loci in gametes and requires the activity of *de novo* as well as maintenance DNA methyltransferases (Chapter 1 **Figure 1.4.1.2**).

In contrast, imprinting in plants is likely to involve the sex-specific de-methylation of loci and appears to require only maintenance DNA methyltransferase activity. In support of this, genomes inherited from the *de novo* methyltransferases mutant *drm1drm2* and the non-GC maintenance DNA methyltransferase mutant *cmt3* do not induce parent-of-origin-specific affects on seed development or loss of FWA imprinting (Kinoshita, T. et al. 2004, Xiao, W. et al. 2006a). This indicates that *de novo* and non-CG maintenance DNA methyltransferases plays a role in imprinting (Kinoshita, T. et al. 2004, Xiao, W. et al. 2006a). *MET2a*, *MET2b* and *MET3* have remained the only putative DNA methyltransferases to have a potential role in genomic imprinting. It was speculated that these genes could have a tissue-specific role comparable to the isoforms of DNMT1 in mammals. Contrary to this hypothesis, MET2a, MET2b and MET3 play no detectable role in imprinting, which defines MET1 the sole DNA methyltransferase required for imprinted. It is possible that alternative splicing of MET1 transcript generates functionally distinct isoforms of *MET1* but to date there is no evidence to support this.

3.3.4 *The role of multiple MET1 family genes*

A *MET1* gene family has been identified in a number of plant species including rice, carrot and *Brassica rapa* (Bernacchia, G. et al. 1998, Fujimoto, R. et al. 2006, Teerawanichpan, P. et al. 2004). In *Arabidopsis*, carrot and *Brassica rapa,* the MET1 families are suggested to have arisen from recent gene duplication events (Finnegan, E. J. and Kovac, K. A. 2000, Genger, R. K et al. 1999, Pavlopoulou, A. and Kossida, S. 2007). Phylogenetic analysis indicates that the *Arabidopsis MET1* family genes are more similar to each other than to those of the *Brassica rapa MET1* family, indicating that gene duplication occurred after speciation (Pavlopoulou, A. and Kossida, S. 2007). However, some species, including pea, tobacco and maize, have only a single MET1 gene suggesting that *MET1* duplication is not

favoured in all species (Nakano, Y. et al. 2000, Pradhan, S. et al. 1998, Steward, N. et al. 2000).

In species with a single *MET1* gene, expression is prominent in rapidly dividing tissues (Nakano, Y. et al. 2000, Pradhan, S. et al. 1998, Steward, N. et al. 2000). This is consistent with a role for MET1 in maintaining CG methylation patterns after cell division. In species with a *MET1* gene family, one member appears to be expressed prominently in rapidly dividing tissue, whereas the other member/s are expressed tissue-specifically. For example, in carrot, *DcMET1-5* is more strongly expressed than *DcMET1-2,* and *DcMET1-5* transcript is distributed throughout the apical meristem and in leaf primordia of all ages whereas *DcMET1-2* transcript is restricted to a small group of cells in the youngest leaf primordia (Bernacchia, G. et al. 1998). In *Brassica rapa*, *BrMET1a* is more strongly expressed than *BrMET1b,* and *BrMET1a* is widely expression in vegetative and floral tissue, whereas *BrMET1b* expression is restricted to the pistil (Fujimoto, R. et al. 2006). Similarly, MET1 is more strongly and broadly expressed than *MET2a* and *MET2b* (**Figure 3.5**). This indicates that duplicated MET1 genes are required for a tissue specific role. Such genes could function in the tissue-specific regulation of gene expression.

In the *Arabidopsis* accession Col-0, MET3 is apparently a pseudogene. However, it is possible that other accessions may encode a functional MET3 DNA methyltransferase. In support of this, the stop codon and deletion identified in the Col-0 *MET3* coding region were not found within the same region of C24 (**3.2.2.2**) or Landsberg *erecta* (La-*er*) (Finnegan, E. J. and K 2000). Moreover, a mutant screen of Ds transposon insertion lines in the accession La-*er*, identified a *MET3* insertion line as a maternal embryo arrest mutant (Pagnussat, G. C. et al. 2005). However, it has not been confirmed that this phenotype was caused by MET3 loss-of-function and not a mutation in a linked gene.

Despite strong evidence suggesting that *MET3* is a pseudogene, AtGenExpress microarray data shows that *MET3* transcript is present at a low level in many tissues in the *Arabidopsis* accession Col-0. Additionally, Ka/Ks ratio analysis indicated that MET3 putative functional domains have been conserved by purifying selection. Pseudogene transcription has been reported in many species. For example, in *Arabidopsis,* the myrosinase pseudogene *TGG3* is expressed in stamens and petals (Zhang, J. et al. 2002). The evolutionary conservation of pseudogene sequence has also been reported in many species

suggesting that some pseudogene may have a functional role (Balakirev, E. S. and Ayala, F. J. 2003). One hypothesis suggests that pseudogene genes could provide a source of antisense RNA to regulate the expression of homologous genes (McCarrey, J. R. and Riggs, A. D. 1986). It is therefore possible that *MET3* has role other than DNA methyltransferase activity. Alternatively, *MET3* may have become a pseudogene recently and therefore has had too little time to be further altered by deleterious mutants.

Chapter 4 – *Tools to analyse the role of MET1 in genomic imprinting*

4.1 *Introduction*

As discussed in Chapter 1 (section **1.4.2.5**), the current model of DNA methylation-dependent imprinting in plants has significant weaknesses. For example, the model describes a mechanism to control the expression of maternally-expressed/paternally-silenced imprinted genes. However, the large seed phenotype of [*MET1*as x WT] and [*met1-9* x WT] seed indicates the existence endosperm-promoting genes that are paternally-expressed/maternal-silenced. The current model therefore only explains how expression of a proportion of imprinted genes is controlled.

In an attempt to understand how imprinting of a broader range of genes is controlled, two novel imprinting models were proposed; these differ in their requirement for MET1 during the gametophyte generation (Chapter 1, section **1.4.2.6**). Briefly, both models require that the default state of imprinted genes is methylated and silent and that imprinting is achieved by the sex-specific demethylation of alleles. Both models propose that MET1 is required throughout sporophytic development to maintain the default methylated state of imprinted loci and model 1 proposes that MET1 is also required throughout the gametophyte generation to maintain methylation of silenced alleles (Chapter 1, **Figure 1.5**). In contrast, model 2 proposes that imprinted loci are sex-specifically 'marked' during to sporogenesis by an epigenetic modification which ensures they are silenced in the endosperm. The model proposes this 'mark' is maintained during the gametophyte generation independently of MET1 and therefore MET1 is not required during the gametophyte generation to maintain imprinting.

As suggested in Chapter 1 (section **1.4.2.7**), the validity of these models can be tested by determining when MET1 is required for imprinting. To this end, the aim of the work described in this chapter was to analyse the expression profile of *MET1* and to develop tools to test when MET1 is required for imprinting.

4.2 *Results*

4.2.1 *The production of* MET1*::*GFP *lines to investigate* MET1 *expression*

The expression profile of MET1 was analysed using *MET1*::*GFP* reporters. This section describes the design, production and preliminary analysis of *MET1*::*GFP* reporter lines in *Arabidopsis*.

4.2.1.1 *The choice of* MET1 *promoter sequences*

Two putative *MET1* promoter sequences were chosen to drive expression of a *GFP* reporter. The *sMET1* promoter sequence consists of 2509 bp immediately upstream of the native *MET1* translational start (**Figure 4.1**). This sequence was previously used to drive a *GUS* reporter and is therefore known to include sequence motifs sufficient to drive expression (Adam, S. 2003). The *lMET1* promoter sequence consists of 1911 bp immediately upstream and 1638 bp downstream of the *MET1* translational start (**Figure 4.1**). This downstream region was included to incorporate the first *MET1* intron because introns reportedly increase transcript stability (Mascarenhas, D. et al. 1990). This has been demonstrated by comparing the activity of *GUS* driven from various promoter sequences of the trytrophan pathway gene *PAT1*. Plants transformed with *PAT1* promoter-*GUS* constructs which include *PAT1* introns, have 30 times higher GUS activity than those transformed with constructs lacking these introns (Rose, A. B. and Last, R. L. 1997).

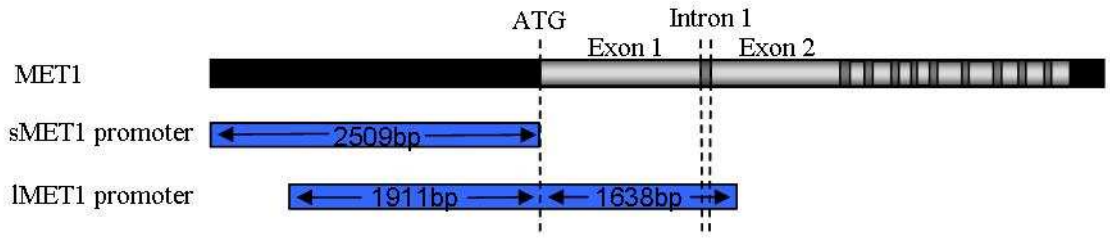


Figure 4.1 Schematic of sMET1 and sMET1 promoter regions in relation to the MET1 genomic sequence

4.2.1.2 *Construction of the* sMET1*::*GFP *and* lMET1*::*GFP *reporters*

sMET1 and *lMET1* were amplified from the TAC vector K2JP3 (www.arabidopsis.org) by PCR using primers SM1::F & SM1::R and LSBM1F & LM1::R respectively. *sMET1* and *lMET1* PCR fragments were ligated into pGEM-T to produce *sMET1*::pGEMT and *lMET1*::pGEMT respectively (**Figure 4.2a** and **b)**. Following transformation into *E. coli,* plasmid from a positive colony for each construct was sequenced. No sequence errors were

detected in the coding region of the *lMET1* sequence or within the first 500 bp upstream of the *MET1* translational start of the *lMET1* and *sMET1* sequences. The *lMET1* and *sMET1* fragments were cut from pGEMT by *Sal*I and *Xba*I ligated into a *Sal*I- and *Xba*I-cut pBI(*dme* NLS-*GFP*) expression vector to produce *sMET1*::*GFP* and *lMET1*::*GFP* constructs respectively (**Figure 4.2c** and **d**).

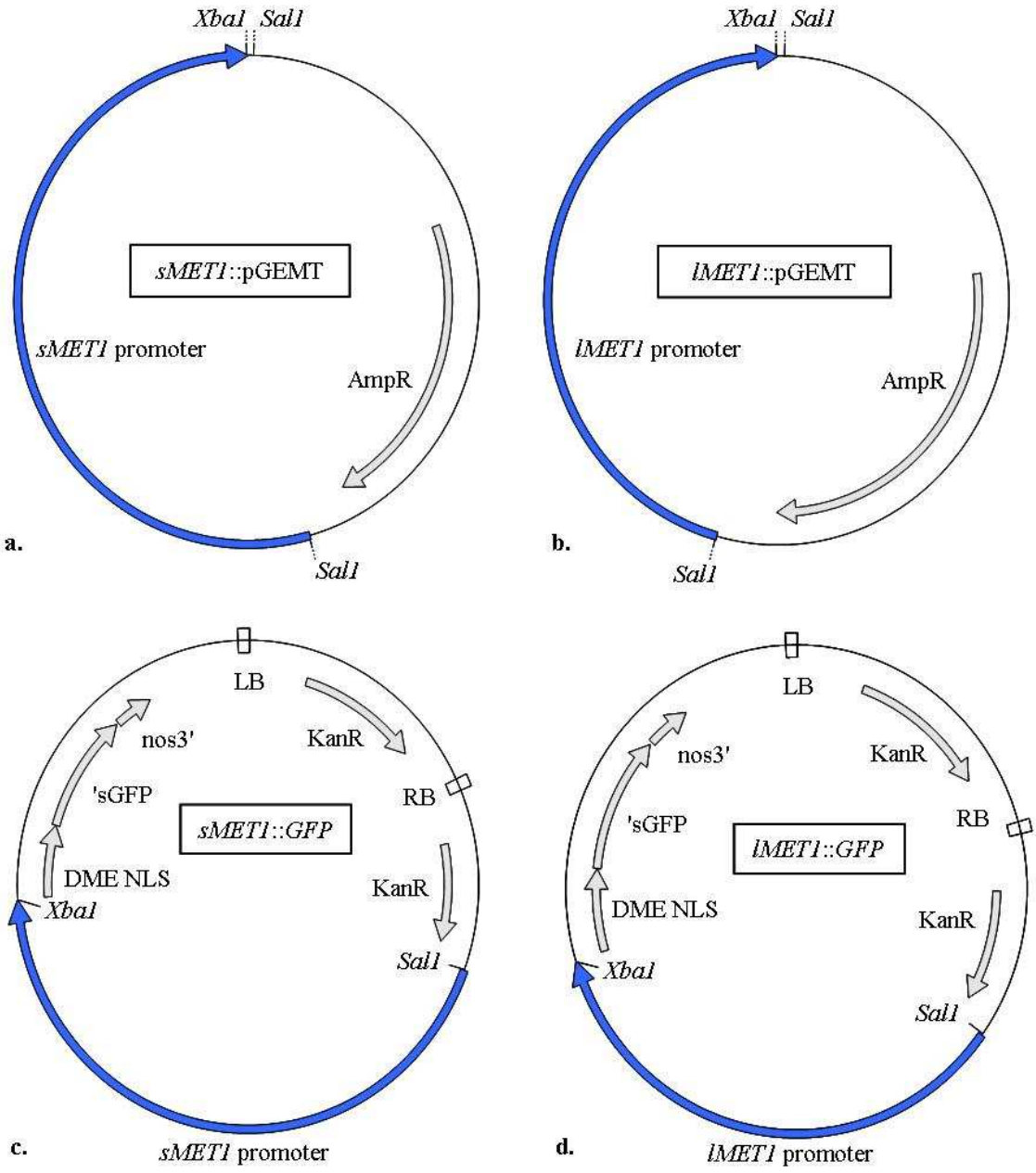


Figure 4.2 Plasmid maps of *sMET1*::*pGEMT* (a), *lMET1*::*pGEMT* (b), *sMET1*::*GFP* (c) and *lMET1*::*GFP* (d).

4.2.1.3 *Transformation of the* sMET*::*GFP *and* lMET1*::*GFP *transgenes into* Arabidopsis

lMET1::*GFP* and *sMET1*::*GFP* constructs were transformed into *A. tumefaciens* and subsequently the transgenes were transformed into the *Arabidopsis* accession *Col-0* by floral dipping. T1 seed was germinated on plant media containing kanamycin to select transgenic lines. Kanamycin-resistant plants were then checked for the transgene presence using PCR. Ten T1 *lMET1*::*GFP* and eleven T1 *sMET1*::*GFP* lines were identified.

4.2.1.4 *Preliminary analysis of* sMET1*::*GFP *and* lMET1*::*GFP *lines*

3DAP seed from each T1 *lMET1*::*GFP* and s*MET1*::*GFP* line was analysed for *GFP* expressed using fluorescence microscopy. Nuclear targeted GFP was observed in the endosperm or integument layers of seed from all lines confirming that both the *lMET1*::*GFP* and s*MET1*::*GFP* transgenes were expressed. Notably, the level of *GFP* expression in s*MET1*::*GFP* lines was considerably higher that detectable in *lMET1*::*GFP* lines.

The three s*MET1*::*GFP* and *lMET1*::*GFP* lines with the highest *GFP* expression level were selected for further analysis; these were s*MET1*::*GFP* lines 5, 13 and 14 and *lMET1*::*GFP* lines 3, 5 and 9. To determine the number of transgene insertion per line, T2 seed was germinated on kanamycin plates. s*MET1*::*GFP* lines 5, 13 and 14 and *lMET1*::*GFP* lines 3 and 5 displayed an approximate 3:1 resistant: non-resistant ratio indicating they carry a single copy of the transgene. *lMET1*::*GFP* line 9 displayed a higher resistant: non-resistant ratio suggesting that this line carries multiple copies of the transgene.

4.2.2 GFP *expression in* sMET1*::*GFP *lines*

sMET1::*GFP* lines 5, 13 and 14 were analysed for *GFP* expression within tissues of interest to determine when *MET1* is expressed. Of particular interest, is the expression profile of *MET1* during anther and carpel development and in the male and female gametophyte generation because imprinted loci must be sex-specifically and epigenetically modified at some stage in this developmental window. The absence of *MET1* expression in this window could highlight a period when imprinted loci can become passively demethylated or when *MET1* is not required for imprinting. Conversely, if *MET1* is expressed constitutively in this window, it would indicate that MET1 is potentially available to maintain imprinting-dependent methylation. Additionally, the *sMET1*::*GFP*

reporter lines were used to analyse parent-of-origin-specific *MET1*expression during early seed development, with the aim of determining how methylation at imprinted loci is maintained during embryo and endosperm.

4.2.2.1 sMET1*::*GFP *expression during anther development and the male gametophyte generation*

Developing anthers and petals within floral buds from *sMET1*::*GFP* lines were analysed for *GFP* expression using fluorescence microscopy (**Figure 4.3**). Buds were staged as described by Smyth et al (1990). GFP was present in anther primordia and in developing anthers from *sMET1*::*GFP* lines at all floral stages until around stage 11-12 (**Figure 4.3a, b, e** and **f**). At late floral stage 12, just prior flower opening, GFP was absent in the anther wall but remained detectable in the filament (**Figure 4.3g** and **h**). Additionally, GFP was present in petals at all floral stages before flower opening (**Figure 4.3c** and **d**).

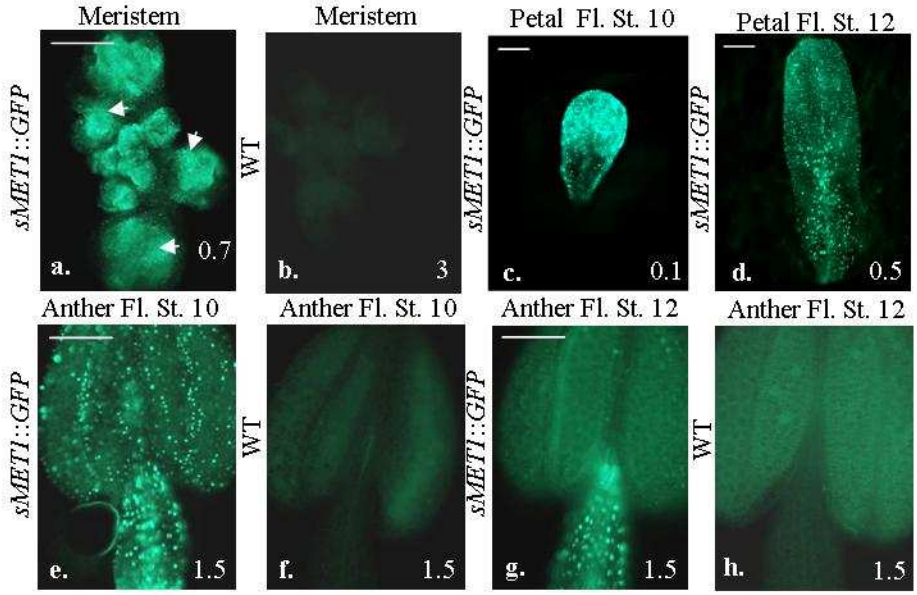


Figure 4.3 *sMET1*::*GFP* expression during anther and petal development. a. GFP in the inflorescence meristem and young buds from *sMET1*::*GFP* lines. Arrows point to anther primordia and developing anthers. **b**. Auto-fluorescence from a WT inflorescence meristem and young buds. **c.** and **d.** GFP in petals from *sMET1*::*GFP* lines at floral stage 10 and 12 respectively. **e.** and **g.** GFP in anthers from *sMET1*::*GFP* lines at floral stage 10 and 12 respectively. **f.** and **h.** Auto-fluorescence from WT anthers at floral stage 10 and 12 respectively. The number in the bottom right hand corner of the images indicates the exposure time of the photographs in seconds.

sMET1::*GFP* lines were analysed for *GFP* expression throughout the male gametophyte generation using confocal microscopy. Nuclear fluorescence was observed in microspore mother cells and uni- and bi-nucleate pollen from *sMET1*::*GFP* lines (**Figure 4.4a** and **c**). However, using the same fluorescence detection settings, faint nuclear auto-fluorescence was observed in microspore mother cells and uni- and bi-nucleate pollen from WT plants (**Figure 4.4b** and **d**). In tri-nucleate pollen, GFP was detected in generative and sperm nuclei from *sMET1*::*GFP* lines and no auto-fluorescence in the GFP channel was detected from WT pollen (**Figure 4.4e** to **j**).

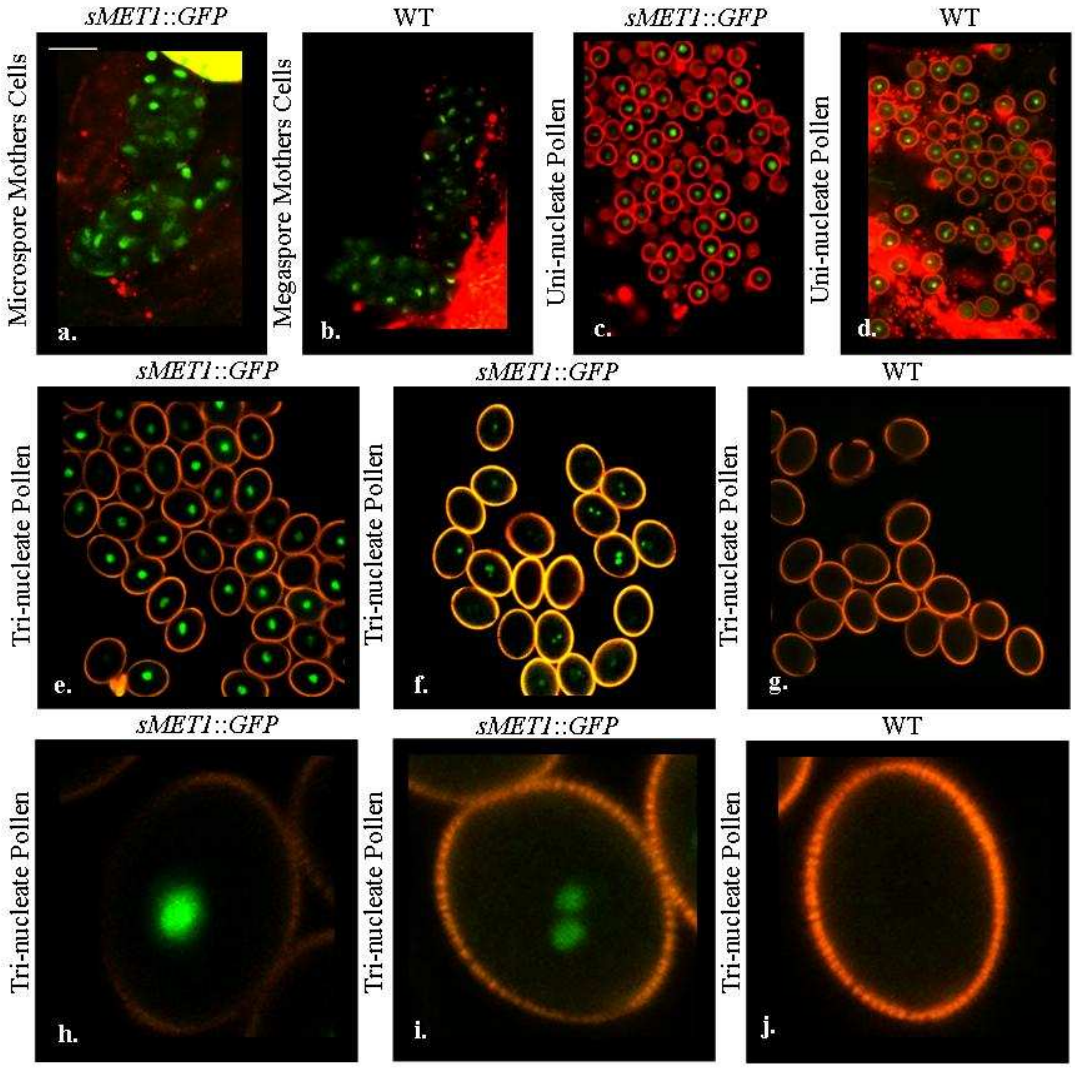


Figure 4.4 *sMET1*::*GFP* expression in the male gametophyte generation. a. and **b.** Fluorescence from a column of microspore mother cells from *sMET1*::*GFP* and WT plants respectively. **c.** and **d.** Fluorescence from uni-nucleate pollen from *sMET1*::*GFP* and WT plants respectively. **e.** and **f.** GFP in the generative nuclei and sperm cells of tri-nucleate from *sMET1*::*GFP* plants **g.** Fluorescence from WT tri-nucleate pollen. **h. i.** and **j.** show an enlarged pollen grain from **e. f.** and **g.** respectively. Scale bar, 25 μm.

4.2.2.2 sMET1*::*GFP *expression during carpel development and the female gametophyte generation*

Developing carpels within the floral buds from *sMET1*::*GFP* lines were analysed for *GFP* expression using fluorescence microscopy. Strong *GFP* expression was observed in carpel meristems and developing carpels at all floral stages prior to flower opening (**Figure 4.5**).

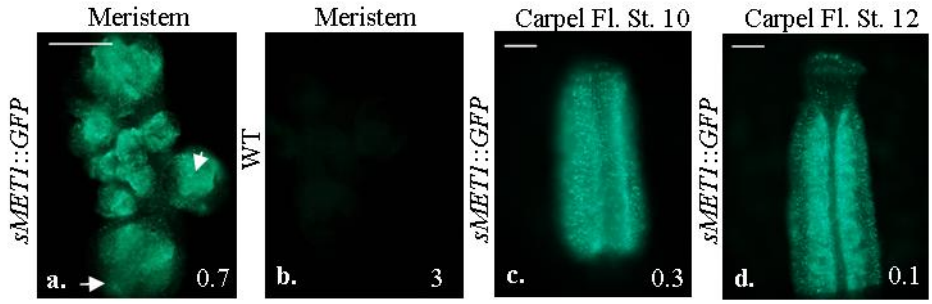
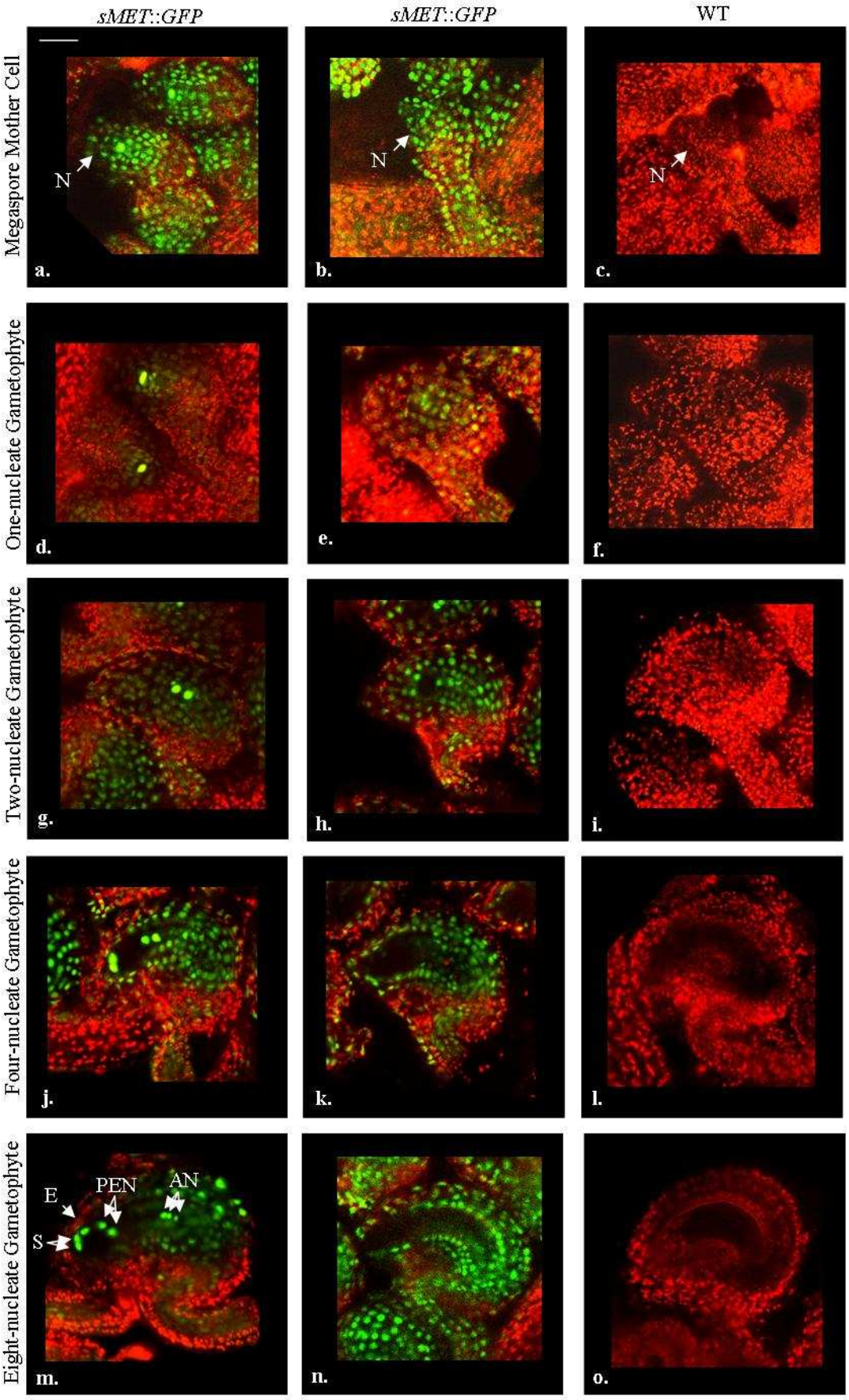


Figure 4.5 ***sMET1*::*GFP*** **expression during carpel development. a.** GFP in the inflorescence meristem and young buds from *sMET1*::*GFP* lines. Arrows point to carpel primordia and developing carpels. **d**. Auto-fluorescence from a WT inflorescence meristem and young buds. **c.** and **d.** GFP in carpels from *sMET1*::*GFP* lines at floral stage 10 and 12 respectively. The number in the bottom right hand corner of the images indicates the exposure time of the photographs in seconds. Scale bar, 100 μm.

sMET1::*GFP* lines were analysed for *GFP* expression throughout the female gametophyte generation using confocal microscopy and developing female gametophytes were staged as described in Christensen *et al* (1997). Analysis of *GFP* expression in the female gametophyte was obscured by *GFP* expression in integument tissue. To address this problem, plants hemizygous for the *sMET1*::*GFP* construct were analysed. 50% of female gametophytes produced by these plants did not inherit the reporter and were used as an internal control for the absence of the reporter. Strong *GFP* expression was detected within the female gametophyte region of half of the ovules; this was assumed to be female gametophyte-specific *GFP* expression (**Figure 4.6**). Strong *GFP* expression was detected within a single cell of the nucellus at FG0 (megasporogenesis) (**Figure 4.6a**); this is likely to be the megaspore mother cell prior to meiosis because the predominant fluorescing cell appears to be in direct contact with the nucellar epidermis and the integument layers have just initiated development. Strong *GFP* expression was also detected in one, two and four nuclei in female gametophytes staged at FG1, FG2 and FG4 respectively (**Figures 4.6d, g** and **j**). This indicates that the reporter is expressed in one-, two- and four-nucleate

gametophytes. Analysis of *GFP* expression in female gametophytes at FG5-7 was severely hindered by strong fluorescence from the integuments but GFP was nevertheless detectable in four to five nuclei at the micropylar end of some embryo sacs. Given their relative position, these are most likely two synergids, the eggs cell and either the two primary endosperm nuclei or the central cell after fusion of the primary endosperm nuclei (**Figure 4.6m**)

Figure 4.6 *sMET1*::*GFP* expression during the female gametophyte generation. Columns 1 and 2 show female gametophyte development in hemizygous *sMET1*::*GFP* plants. Column 1 shows gametophytes assumed to have inherited the *sMET1*::*GFP* reporter and column 2 shows control gametophytes which show no sign of reporter expression. Column 3 shows female gametophyte from WT plants. **a. b.** and **c.** Ovules at FG0 (megasporogenesis). **d. e.** and **f.** Ovules at FG1 (one-nucleate stage). **g. h.** and **i.** Ovules at GF2 (two-nucleate stage). **j. k.** and **l.** Ovules at FG4 (four-nucleate stage). **m. n.** and **o.** Ovules at FG5 (eight-nucleate stage). Positions of the nucellus epidermis (N), synergids (S), egg cell (E), primary endosperm nuclei (PEN) and antipodal nuclei (AN) are suggested. Scale bar, 25 μm.


sMET::GFP
sMET::GFP
WT
Megaspore Mother Cell
One-nucleate Gametophyte
Two-nucleate Gametophyte
Four-nucleate Gametophyte
Eight-nucleate Gametophyte
N
E
PEN
AN
S
a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.
l.
m.
n.
o.

4.2.2.3 *Parent-of-origin-specific* sMET1*::*GFP *expression during early seed development*

The parent-of-origin-specific expression of the *sMET*::*GFP* reported was analysed in developing seed to investigate how methylation at imprinted loci is maintained during early embryo and endosperm development.

1, 2 and 3 DAP seed from reciprocal *sMET*::*GFP* line 13 X WT crosses was analysed for *GFP* expression using confocal microscopy. When the *sMET1*::*GFP* reporter was inherited maternally, GFP was detected in the dividing endosperm syncitium at 1, 2 and 3 DAP (**Figure 4.7a** and **d**). Prior to 3DAP it was difficult to detect *GFP* expression in the embryo but at 3DAP GFP in the embryo was unambiguous (**Figure 4.7e**).

When the *sMET1*::*GFP* reporter was inherited paternally GFP was present in the dividing endosperm syncitium at 1DAP (**Figure 4.7f and g**). However, at 2DAP the signal became very difficult to detect and only faint signal was present in the peripheral and chalazal endosperm of some seed (**Figure 4.7h**). At 3DAP GFP was restricted to the embryo (**Figure 4.7i**).

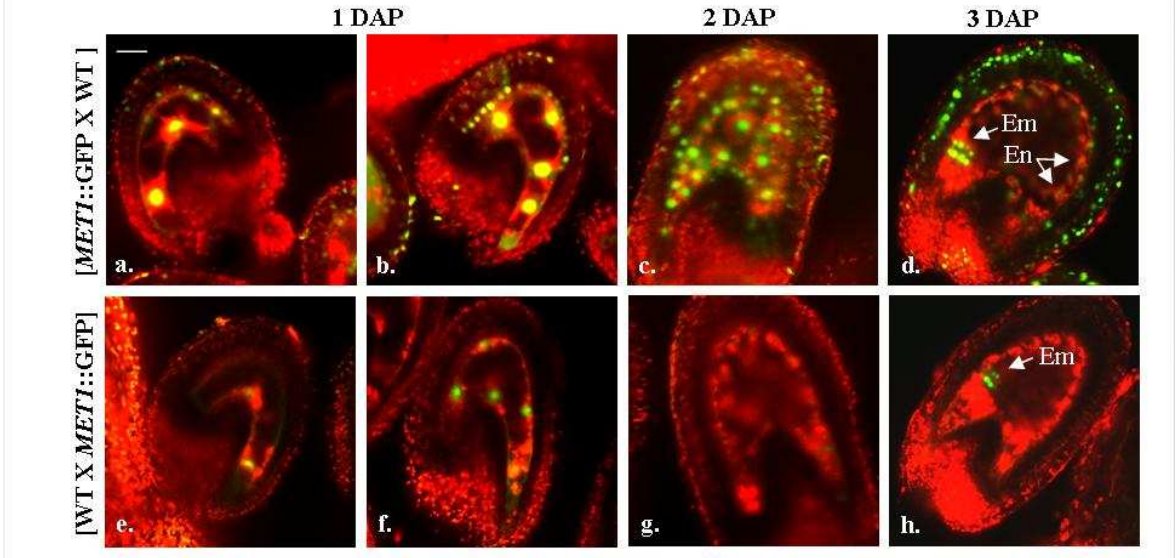


Figure 4.7 Parent-of-origin-specific *sMET1*::*GFP* expression. This figure is organised with [*sMET*1::*GFP* X WT] seed on the top row and [WT X *sMET1*::*GFP*] seed on the bottom row. **a. b. e.** and **f.** Seed at 1DAP. **c.** and **g.** Seed at 2DAP. **d.** and **h.** Seed at 3DAP. Arrows point to embryo (Em) and endosperm (En) nuclei. Scale bar, 25 μm.

4.2.3 *Analysis of* l**MET1*::*GFP *lines***

T2 *lMET1*::*GFP* plants were grown with the aim of further investigating the *MET1* expression profile using fluorescence microscopy. However, the aim of this work was diverted after observing that some T2 *lMET1*::*GFP* plants displayed interesting phenotype characteristics. In contrast to the WT-like phenotype expected, some kanamycin-resistant T2 *lMET1*::*GFP* plants had a *met1*-like phenotype; *met1*-like *lMET1*::*GFP* plants were late flowering and had more auxiliary flowering shoots and floral homeotic mutations than their WT-like *lMET1*::*GFP* siblings (**Figure 4.8a**). This *met1*-like phenotype was observed in 14/19 *lMET1*::*GFP* line 3 plants, 17/19 *lMET1*::*GFP* line 9 plants and 3/18 *lMET1*::*GFP* line 5 plants. The occurrence of the *met1*-like phenotype in all three *lMET1*::*GFP* lines indicated that the phenotype was not due to disruptive transgene insertion events

To investigate whether both *met1*-like and WT-like *lMET1*::*GFP* plants inherited the *lMET1*::*GFP* transgene, PCR was used to test for the presence of the transgene in WT-like and *met1*-like plants from *lMET1*::GFP lines 3, 9 and 5. The reporter construct was detected in both classes of plant indicating that the presence of the *lMET1*::*GFP* transgene is not diagnostic of the *met1*-like phenotype (**Figure 4.8b**).

One possible explanation for the *met1*-like phenotype is that the *lMET1*::*GFP* transgene causes *MET1* cosuppression. Cosuppression is transgene-induced reduction of homologous endogenous transcripts (Napoli, C. et al. 1990). As the *lMET1* promoter includes a fragment of the *MET1* coding region, it is possible that *lMET1*::*GFP* expression induces a reduction in the steady-state level of *MET1* transcript which in turn causes hypomethylation and associated phenotype abnormalities. To test this possibility, semi-quantitative RT PCR was used to compare the level of *MET1* transcript in WT-like and *met1*-like *lMET1*::*GFP* plants. A much lower level of *MET1* transcript was detected in the *met1*-like *lMET1*::*GFP* plants compared with the WT-like *lMET1*::*GFP* and WT plants analysed (**Figure 4.8b**). Additionally, a slightly lower level of *MET1* transcript was detected in the WT-like *lMET1*::*GFP* plant compared with the WT plant analysed.

To investigate the methylation status of *lMET1*::*GFP* plants, RT PCR was used to test for ectopic expression of *FWA* in leaf tissue from WT-like and *met1*-like *lMET1*::*GFP* plants and WT plants. Consistent with the late flowering phenotype, ectopic *FWA* expression was

detected in *met1*-like *lMET1*::*GFP* plants indicating that these plants are hypomethylated (**Figure 4.8c**). These findings indicate that *lMET1*::*GFP* expression can cause *MET1* cosuppression and a high level of suppression can cause hypomethylation and phenotype abnormalities.

GFP expression from *lMET1*::*GFP* lines was very low and difficult to detect in tissues other than the endosperm. For this reason these lines were not used to analyse the expression profile of *MET1*.

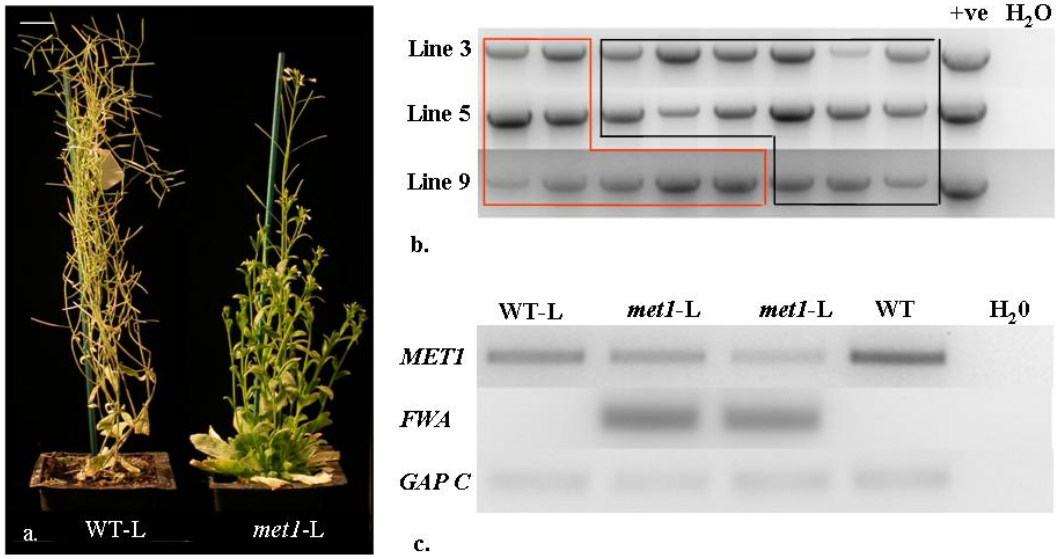


Figure 4.8 Phenotype and molecular analysis of *lMET1*::*GFP* lines. a. WT-Like (WT-L) and *met1*-like (*met1*-L) *lMET1*::*GFP* plants. **b.** A fragment of the *lMET1*::*GFP* transgene amplified by PCR from eight plants of *lMET1*::*GFP* lines 3, 5 and 8. The red box highlights plants with a *met1*-like phenotype and the black box highlights plants with a WT-like phenotype. **c.** Semi quantitative RT-PCR comparing *MET1* and *FWA* transcript levels in leaf tissue from WT-like and *met1*-like plants. A fragment of the *GAPC* gene was amplified as a control. Scale bar, 2 cm.

4.2.4 *Tools to suppress* MET1 *expression*

The approach chosen to investigate when MET1 is required for imprinting was to suppress *MET1* tissue specifically and test for altered imprinting; it was therefore necessary to develop an approach to suppress *MET1* which can be adapter to use tissue-specifically. This section investigates whether *MET1* can be suppressed by RNA interference (RNAi); an endogenous gene silencing mechanism. This targeted gene silencing approach was chosen because in comparison with others, including antisense suppression and co-

suppression, RNAi is believed to be superior due of its high level of efficiency and stability (Jones, L. et al. 2001, Kusaba, M. 2004). RNAi is induced by the cleavage of double stranded (ds) RNA into short RNAs. These are incorporated in RNAi-induced silencing complexes (RISCs) and can induce post-transcriptional gene silencing (TGS), by targeting complementary mRNA sequences for degradation, or transcriptional gene silencing (TGS) which is associated with the RNA-directed DNA methylation (RdDM) of complementary DNA sequences (Baulcombe, D 2004, Dorokhov, Y. L. 2007). Transgene inverted repeats (IR), which when expressed produce dsRNA, can also trigger RNAi-induced TGS and RNAi-induced PTGS of endogenous sequences (Mourranin, P. et al. 2007). Research to date suggests that PTGS is triggered by dsRNA which contain sequences homologous to the coding regions whereas TGS is triggered by dsRNA which contain sequences homologous to promoter regions.

There were some concerns that *MET1* would be immune to RNAi-induced TGS because MET1 is reportedly required for the establishment and maintenance of RdDM. For example, TSG of a *NOS*::*NPT11* transgene is associated with methylation of the *NOS* promoter and is induced in the presence on *NOS* promoter IR (Aufsatz, W. et al. 2002). However, in *met1* mutants, this methylation is incomplete (Aufsatz, W. et al. 2004). Additionally, silencing of a fully methylated *NOS*::*NPTII* transgene introduced into a *met1* mutant background is not maintained despite the continued presence of the silencing signal from the *NOS* promoter IR (Aufsatz, W. et al. 2004). Similarly, maintenance of RdDM methylation and silencing of a *35S*::*GFP* transgene is lost in plants with suppressed *MET1* expression (Jones, L. et al. 2001). For this reason, it was decided that *MET1* suppression should be attempted by RNAi-induced PTGS, i.e. by producing transgenic lines expressing a transgene IR encoding a sequence homologous to the coding region of *MET1*.

4.2.4.1 *ChromDB* MET1-*RNAi lines*

Following the decision to use RNAi-induced PTGS to suppress *MET1*, it became apparent that *Arabidopsis MET1*-RNAi lines are available from the Plant Chromatin Database (ChromDB) (http://www.chromdb.org/). The ChromDB *MET1*-RNAi lines carry a transgene encoding a CaMV *35S* promoter driving a single copy IR of the first 564 bp downstream of the native *MET1* transcriptional start. It was predicted that the expression of this transgene would trigger RNAi-induce PTGS of *MET1* and induce genome-wide hypomethylation at CG sites. As exemplified by the phenotype of the *met1-9* line, loss of

MET1 expression and CG hypomethylation causes numerous obvious phenotype abnormalities including delayed flowering (Chapter 3, section **3.2.5.2**). Consequently, a functional ChromDB *MET1*-RNAi line should be late flowering and display other typical abnormalities associated with hypomethylation. This section describes the analysis of five ChromDB *MET1*-RNAi lines, namely CS30941, CS30941, CS30943, CS30945 and CS30946.

ChromDB *MET1*-RNAi lines were grown alongside WT controls. Contrary to the predictions, ChromDB *MET1*-RNAi lines were not late flowering and were phenotypically indistinguishable from WT plants. To test whether the ChromDB *MET1*-RNAi lines carry the *MET1*-RNAi transgene, a selection of ChromDB *MET1*-RNAi plants were analysed for the presence of a 5' region of the transgene by PCR. The transgene was detected in all plants analysed. Subsequently, to test whether *MET1* is suppressed in ChromDB *MET1*-RNAi lines, semi-quantitative RT PCR was used to compare *MET1* transcript level in leaf tissue from *MET1*-RNAi lines CS30943 and CS30945 and a WT control plant. No difference in *MET1* transcript level was detected between the ChromDB *MET1* RNAi lines and the WT plant analysed (**Figure 4.9**). This indicates that *MET1* is not suppressed in the ChromDB *MET1*-RNAi lines.

Figure 4.9 Semi-quantitative RT-PCR analysing the *MET1* transcript level in ChromDB *MET1*-RNAi lines. A fragment of the *GAPC* gene was amplified as a control.

There are several reasons why the ChromDB *MET1*-RNAi lines appear to display no evidence of *MET1* suppression including the possibility that the transgene is corrupt and non-functional or not expressed. It is also possible that the transgene is expressed and functional but does not reduce *MET1* levels sufficiently to induce hypomethylation and associated phenotypic abnormalities. Alternatively, it was possible that *MET1* can not be suppressed by RNAi-induced PTGS. In support of this latter suggestion, it is reported that MET1 may be required to maintain RNAi-induced PTGS. RNAi-induced PTGS is correlated with coding sequence methylation (Baulcombe, D. C. 1996) and it has been

suggested that the maintenance of this methylation is required to retain the chromatin state necessary for aberrant RNA synthesis (Vaucheret, H. et al. 2001). Moreover, RNAi-induced PTGS of a 35S::GUS direct repeat transgene is stochastically inhibited in homozygous *met1* mutants (Morel, J. et al. 2000). It is therefore possible that the involvement of MET1 in RNAi-induced PTGS could prevent *MET1* suppression using this approach. To further investigate whether *MET1* can be silenced by RNAi-induced PTGS, transgenic lines carrying a novel *MET1*-RNAi construct were produced and their effectiveness was tested.

4.2.5 *Design, construction and analysis of novel* 35S*::*MET1*-RNAi lines*

The section describes the production of novel *35S*::*MET1*-RNAi lines and an analysis of their methylation status and imprinting ability.

4.2.5.1 *The choice of IR sequence*

For the design of a *MET1*-RNAi construct it was first necessary to select a fragment of the *MET1* coding region to use for an IR to generate dsRNA. To reduce the possibility of off-target effects on other *MET1* family genes, the *MET1* fragment chosen has low homology to the corresponding region of other *MET1* family genes. This fragment is 215 nucleotide in length and includes a 34 nucleotide sequence unique to *MET1* (Figure **4.10**).

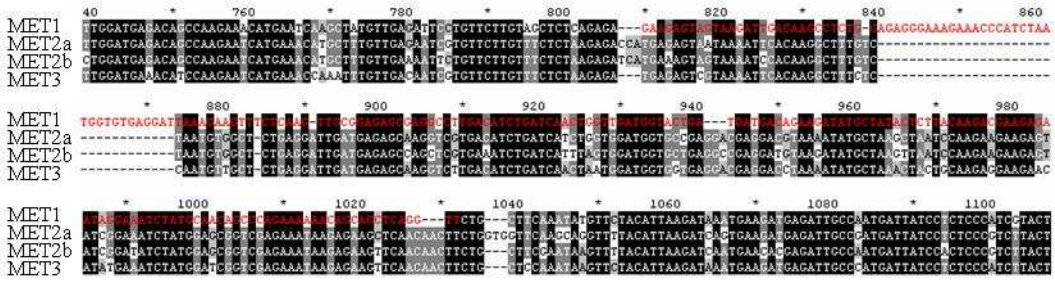


Figure 4.10 The *MET1* fragment selected to use as an IR to trigger RNAi-induced PTGS of *MET1*. An alignment of a fragment of the *MET1* family gene sequences. Red highlights the 215 bp *MET1* fragment containing a 34 nucleotide sequence unique to *MET1*.

4.2.5.2 *Construction of the* 35S*::*MET1*-RNAi constructs*

The 215 bp *MET1* fragment was amplified from WT genomic DNA by PCR using primers LSB56.F and LSB57.R. The 215 bp *MET1* PCR fragment was then ligated into the pGEMT to produce *MET1*-RNAi-pGEMT (**Figure 4.11a**). Following transformation into

E. coli, plasmid from a positive colony was sequenced. No sequence errors were detected. The *MET1* fragment was cut from *MET1*-RNAi-pGEMT by *Asc*I and *Swa*I and ligated in the sense-orientation into *Asc*1- and *Swa*1-cut pFGC5491 to produce *MET1*-RNAi-(sense)-pFGC5491 (**Figure 4.11b**). Subsequently, a second *MET1* fragment was cut from *MET1*-RNAi-pGEMT using *BamH*I and *Xba*I and ligated into *BamH*I- and *Xba*I-cut *MET1*-RNAi-(sense)-pFGC5491 to produce *MET1*-RNAi-(sense & antisense)-pFGC5491 (*35S*::*MET1*-RNAi) (**Figure 4.11c**).

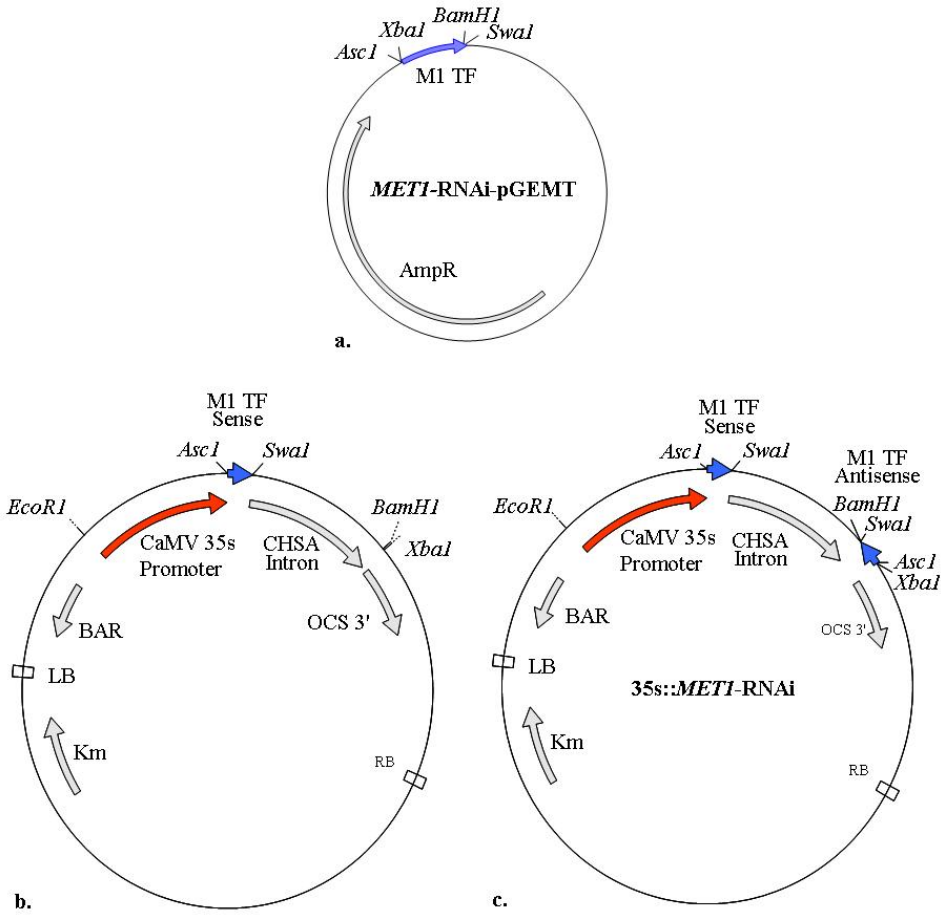


Figure 4.11 Plasmid maps of MET1-RNAi-pGEMT (a), MET1-*RNAi*-(sense)-pFGC5491 (b) and *35S*::*MET1-RNAi* (c).

4.2.5.3 *Transformation of the* 35S*::*MET1-*RNAi* transgene *into* Arabidopsis

The *35S*::*MET1-RNAi* construct was transformed into *A. tumefaciens* and then the transgene was transformed into the *Arabidopsis* accession Col-0 by floral dipping. T1 seedlings were treated with BASTA to select for transgenic lines. BASTA-resistant plants

were further checked for the transgene presence using PCR. The presence of the construct was confirmed in ten *35S*::*MET1-RNAi* T1 plants.

4.2.5.4 *Phenotype analysis of* 35S*::*MET1-*RNAi lines*

As discussed above (section **4.2.4.1.**) RNAi-induced PGTS of *MET1* was expected to induce hypomethylation and its various associated phenotypic abnormalities. To test for *MET1* suppression, the phenotypes of T1 *35S*::*MET1*-RNAi plants were analysed.

All T1 *35S*::*MET1*-RNAi lines displayed phenotypes diagnostic of hypomethylation and were phenotypically similar to *met1-9* plants. For example, 100% (10/10) of the *35S*::*MET1*-RNAi lines were late flowering; several lines produced twice the WT number of rosette leaves before bolting (**Figure 4.12**). Additionally, *35S*::*MET1*-RNAi lines produced more auxiliary flowering shoots and floral homeotic mutations than WT plants. These results indicate that expression of the *35S*::*MET1-RNAi* transgene successfully triggers RNAi–induced PTGS of *MET1* and induces hypomethylation as predicted.

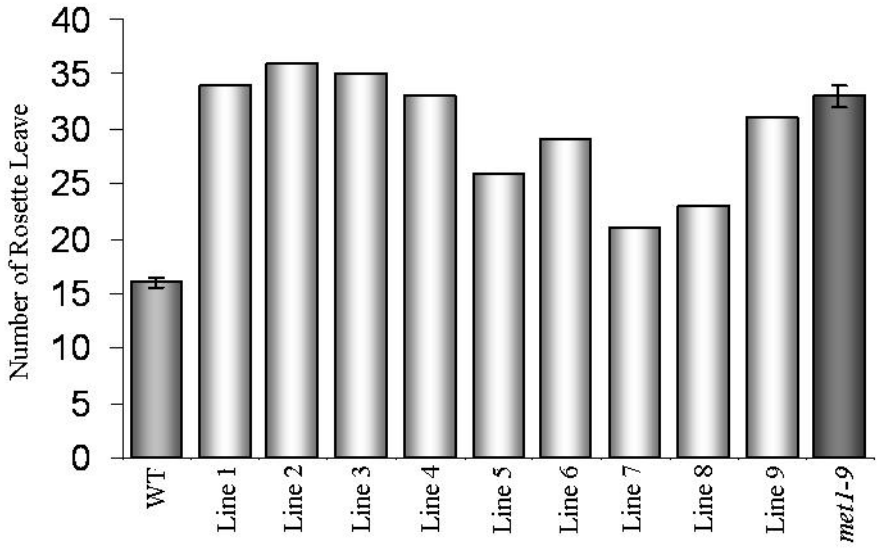


Figure 4.12 The flowering time of T1 *35S*::*MET1-RNAi* lines

4.2.5.5 MET1 *suppression and hypomethylation in* 35S*::*MET1-*RNAi lines*

To further test for *MET1* suppression, semi-quantitative RT-PCR was used to analyse the *MET1* transcript level in leaf tissue from a selection of T1 *35S*::*MET1*-RNAi lines. All lines analysed had a lower *MET1* transcript level than WT plants (**Figure 4.13**). This result

strengthens the suggestion that expression of the *35s*::*MET1*-RNAi transgene results in the transcriptional suppression of *MET1*.

To test whether the *35S*::*MET1*-RNAi transgene induced hypomethylation, semi-quantitative RT-PCR was used to test for ectopic *FWA* expression in leaf tissue from a selection of *35S*::*MET1*-RNAi lines. Ectopic *FWA* expression was detected in all the lines analysed (**Figure 4.13**). This finding indicates that expression of the *35S*::*MET1*-RNAi transgene triggers a sufficient level of *MET1* suppression to induce hypomethylation and release silencing of *FWA*.

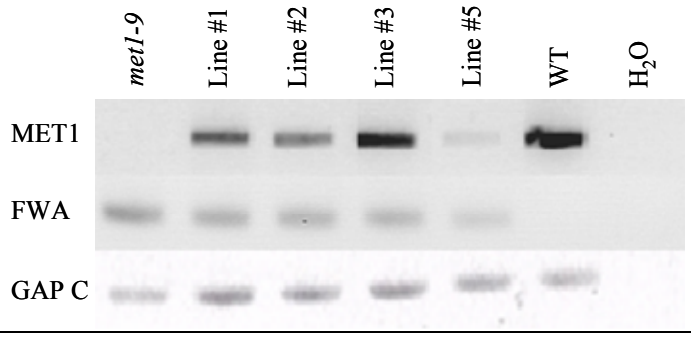


Figure 4.13 Semi-quantitative RT PCR analysing *MET1* and *FWA* transcript levels in leaf tissue from *35S*::*MET1*-RNAi lines. A fragment of the *GAPC* gene was amplified as a control.

4.2.5.6 *Imprinting in* 35S*::*MET1-*RNAi lines*

The analysis described above indicates that expression of the *35S*::*MET1*-RNAi transgene induces *MET1* suppression and hypomethylation. To investigate whether the hypomethylation induced is sufficient to alter imprinted gene expression, imprinting was analysed in several *35S*::*MET1*-RNAi lines using two of the imprinting tests described in Chapter 3 (section **3.2.6.**).

As discussed in previously, the high seed weight of [*MET1as* X WT] and [*met1*-9 X WT] seed and low seed weight of [WT x *MET1as*] and [*met1-9* X WT] seed indicates that loss of *MET1* activity results in the released silencing of maternally-inherited endosperm-promoting genes and paternally-inherited endosperm-inhibiting. To test whether suppression of *MET1* induced in the *35S*::*MET1*-RNAi lines is sufficient to release parent-of-origin-specific silencing of imprinted genes regulating endosperm development, a

selection of these lines were reciprocally crossed with WT plants and the weight of the seed produced from these crosses with compared (**Figure 4.14**).

When the *35S*::*MET1*-RNAi lines were used as seed parents, the weight of seed produced was greater than WT seed, whereas when these lines were used as pollen parents, the weight of seed produced was less than WT seed. These findings indicate that expression of the *35S*::*MET1***-**RNAi transgene suppresses *MET1* sufficiently to release the parent-of-origin-specific silencing of endosperm-inhibiting and -promoting genes.

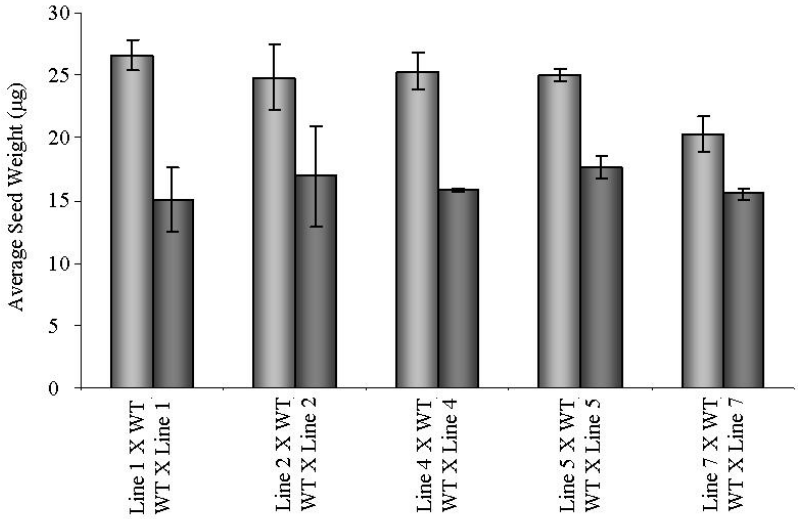


Figure 4.14 Weight of seed produced from reciprocal crosses between *35S*::*MET1*-RNAi lines and WT plants. Average seed weight is calculated as the mean of the average weight of seed from three to five pods per cross.

Imprinting in *35S*::*MET1***-**RNAi lines was further analysed by testing the ability of pollen from *35S*::*MET1***-**RNAi lines to rescue seed abortion of the *mea* mutants. As previously discussed, seed abortion of *mea* mutants is associated with endosperm over-proliferation and hypomethylated pollen is suggested to reduce seed abortion by suppressing this paternal excess phenotype (Chapter 1, **1.4.2.1)**.

mea mutants were emasculated and pollinated by a selection of *35S*::*MET1***-**RNAi lines and the number of shrivelled vs plump seed per pod was scored at 14 DAP. Pollen from all *35S*::*MET1***-**RNAi lines tested dramatically reduced seed abortion of *mea* mutants and four of the five lines reduced seed abortion to a level comparable to that achieved by the *met1-9*

line (**Table 4.1** and **Figure 4.15**). These results provide further evidence that the parent-of-origin-specific silencing of endosperm-inhibiting genes is released in *35S*::*MET1*-RNAi lines and consequently that the *MET1*-RNAi transgene suppresses *MET1* sufficiently to disrupt genomic imprinting. It is therefore suggested that the *MET1*-RNAi transgene will be effective at inducing tissue-specific *MET1* suppression when driven from tissue-specific promoters.

Table 4.1 Percentage seed abortion from crosses between *mea* mutants and *35S*::*MET1*-RNAi lines

Cross[a]	% Seed Abortion[b]	N[c]
mea (La-*er*) X *mea* (La-*er*)	97.0±0.7*	1268
mea (La-*er*) X Line 3 (Col-0)	8.7	23
mea (La-*er*) X Line 5 (Col-0)	43.4	175
mea (La-*er*) X Line 6 (Col-0)	2.1	191
mea (La-*er*) X Line 7 (Col-0)	9.5	189
mea (La-*er*) X Line 10 (Col-0)	4.9	183
mea (La-*er*) X *met1-9* (Col-0)	3.5±1.6*	947

[a] Genetic background of plants shown in brackets
[b] Average seed abortion from one/three pods.
* Mean±S.E.M. of five/six pods from three pollen parent plants
[c] Total number of seeds analysed

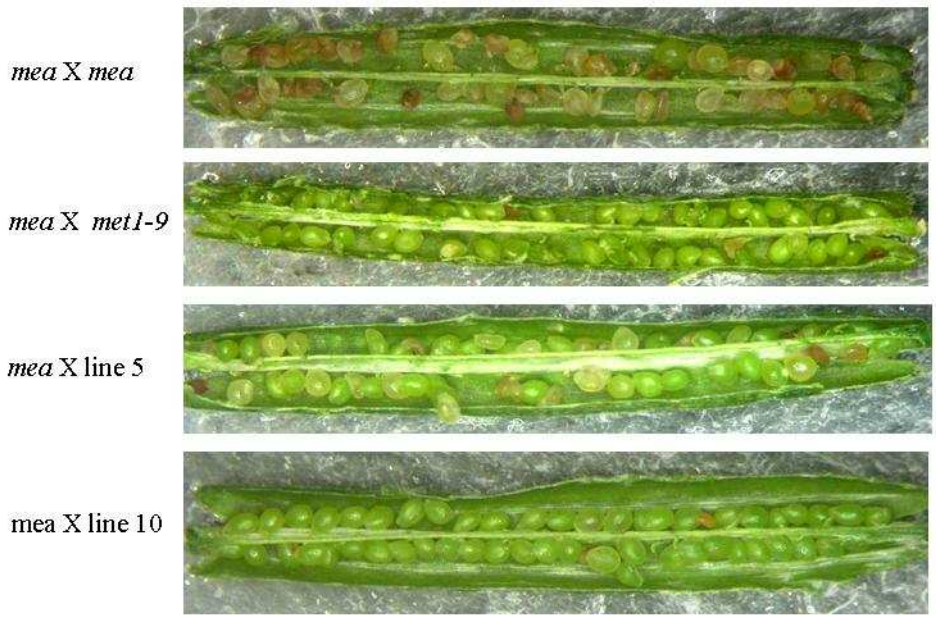


Figure 4.15 Reduced seed abortion of *mea* mutants by pollen from *35S*::*MET1*-RNAi lines. Siliques photographed at 14 DAP

4.2.6 *Tools to signal hypomethylation*

As suggested previously, the approach chosen to investigate when MET1 is required from imprinting was to suppress *MET1* tissue-specifically. The resulting tissue-specific hypomethylation may not induce phenotype abnormalities which are obvious in constitutively hypomethylated lines. It was therefore deemed necessary to develop an approach capable of generating an easily detectable signal of hypomethylation in tissues of interest. To this ends, the aim of the work described in this section was to identify a reporter to signal hypomethylation during anther and carpel development.

An appropriate reporter would be silent as default and expressed in response to hypomethylation, or would display an altered expression pattern in response to hypomethylation. Three imprinted gene promoter-reporter constructs were analysed, namely *FWA*::*GFP*, *MEA*::*GUS* and *mPAC*::*GFP*. The *MEA*::*GUS* reporter was kindly donated by Ueli Grossniklaus (Institute if Plant Biology, Switzerland) and is described in Spillane, et al (2004). The *mPAC*::*GFP* was made by Sushma Tiwari (University of Bath, UK). These reporters were crossed into *met1-9* homozygous plants and their ability to signal hypomethylation was assessed by comparing their expression pattern during floral organ development in a WT and hypomethylated epigenetic background.

4.2.6.1 FWA*::*GFP *expression in WT and* met1-9 *plants*

GFP expression was analysed in WT *FWA*::*GFP*[1] and *met1-9 FWA*::*GFP*[**] plants during anther, carpel and petal development using fluorescence microscopy. Strong fluorescence was detected from the inflorescence meristems and from floral organ primordia of *met1-9 FWA*::*GFP* plants (**Figure 4.16a** and **b**). Additionally, strong fluorescence was detected in developing anthers, carpels and petals of *met1-9 FWA*::*GFP* plants until floral stage 12 (**Figure 4.16c, g** and **i**). In contrast, weak fluorescence was detected in WT *FWA*::*GFP* floral organs throughout development (**Figure 4.16d, e, f, h** and **j**). Initially, this weak fluorescence was assumed to be auto-fluorescence because a comparable level of fluorescence was observed in WT plants. However, increasing the exposure time used to capture digital images from 0.25-0.5s to 2s, revealed that the level of fluorescence from WT *FWA*::*GFP* floral organs is greater than that from WT plants (**Figure 4.16k and l**). These results indicate that the *FWA*::*GFP* reporter is expressed at a high level during floral

[1] The *FWA*::*GFP* reporter in a WT background
[**] The *FWA*::*GFP* reporter in a *met1-9* homozygous background

organ development when in a hypomethylated epigenetic background and at a low level when in a WT background.

To further test for *GFP* expression during floral organ development in WT *FWA*::*GFP* plants, floral organs from these plants were analysed using confocal microscopy. Nuclear targeted *GFP* expression was detected in all floral organs analysed whereas no fluorescence in the *GFP* channel was detected from WT floral organs (date not shown). These results support the finding that the *FWA*::*GFP* reporter is expressed at low levels during floral organ development when in a WT background.

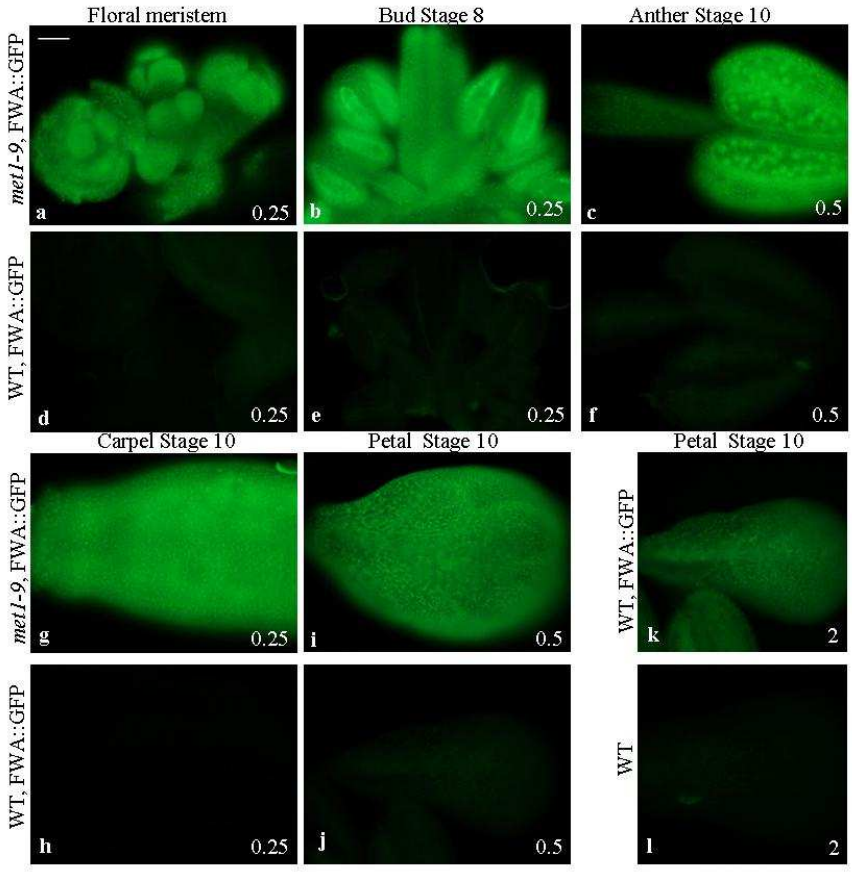


Figure 4.16 *GFP* expression during floral organ development in WT *FWA*::*GFP* and *met-9 FWA*::*GFP* plants. a. and **d.** Fluorescence from meristems of *met1-9* FWA::GFP and WT *FWA*::*GFP* plants respectively. **b.** and **e.** Fluorescence from floral organs from buds at floral stage 8 of *met1-9 FWA*::*GFP* and WT *FWA*::*GFP* plants respectively. **c., g.**, **i., f., h.** and **j.** Fluorescence from anthers, carpels and petals at floral stage 10 of *met1-9 FWA*::*GFP* and WT *FWA*::*GFP* plants respectively. **k.** and **l.** Fluorescence from petals at floral stage 10 of WT *FWA*::*GFP* and WT plants respectively. The number in the bottom right hand corner of images indicates the exposure time of the photographs in seconds. Scale bar, 50 μm.

GFP expression was also analysed during male and female gametophyte development in *met1-9 FWA*::*GFP* and WT *FWA*::*GFP* plants. In *met1-9 FWA*::*GFP* plants, fluorescence in pollen was observed within anthers at floral stage 12 (**Figure 4.17a**). In contrast fluorescence in pollen was not detected within anthers of WT *FWA*::*GFP* plants or WT plants (**Figure 4.17b** and **c).** *GFP* expression in mature pollen was further analysed using confocal microscopy. *GFP* expression was detected in the vegetative nuclei and from the two polar nuclei of mature pollen from *met1-9 FWA*::*GFP* plants (**Figure 4.17d)**. In contrast, no *GFP* expression was detected in pollen from WT *FWA*::*GFP* or WT plants (**Figure 4.17e** and **f**). This indicates that the *FWA*::*GFP* reporter is expressed during male gametogenesis in a hypomethylated epigenetic background and is silent in a WT background.

GFP expression in mature female gametophytes was also analysed using confocal microscopy. The analysis of *GFP* expression within the female gametophyte of *met1-9 FWA*::*GFP* plants was complicated by *GFP* expression in the integument layer. However, *GFP* expression was observed from the central cell and from other cells at the micropylar end of the female gametophyte; these cells are likely to be the egg cell and the synergids (**Figure 4.17g**). In contrast, *GFP* expression was only observed in the central cell of female gametophytes from WT *FWA*::*GFP* plants and no fluorescence within the GFP channel was observed in female gametophytes from WT plants (**Figure 4.17h** and **i**). These results suggest that the *FWA*::*GFP* reporter is more broadly expressed in female gametophytes from a hypomethylated epigenetic background and has central cell-specific expression in a WT background.

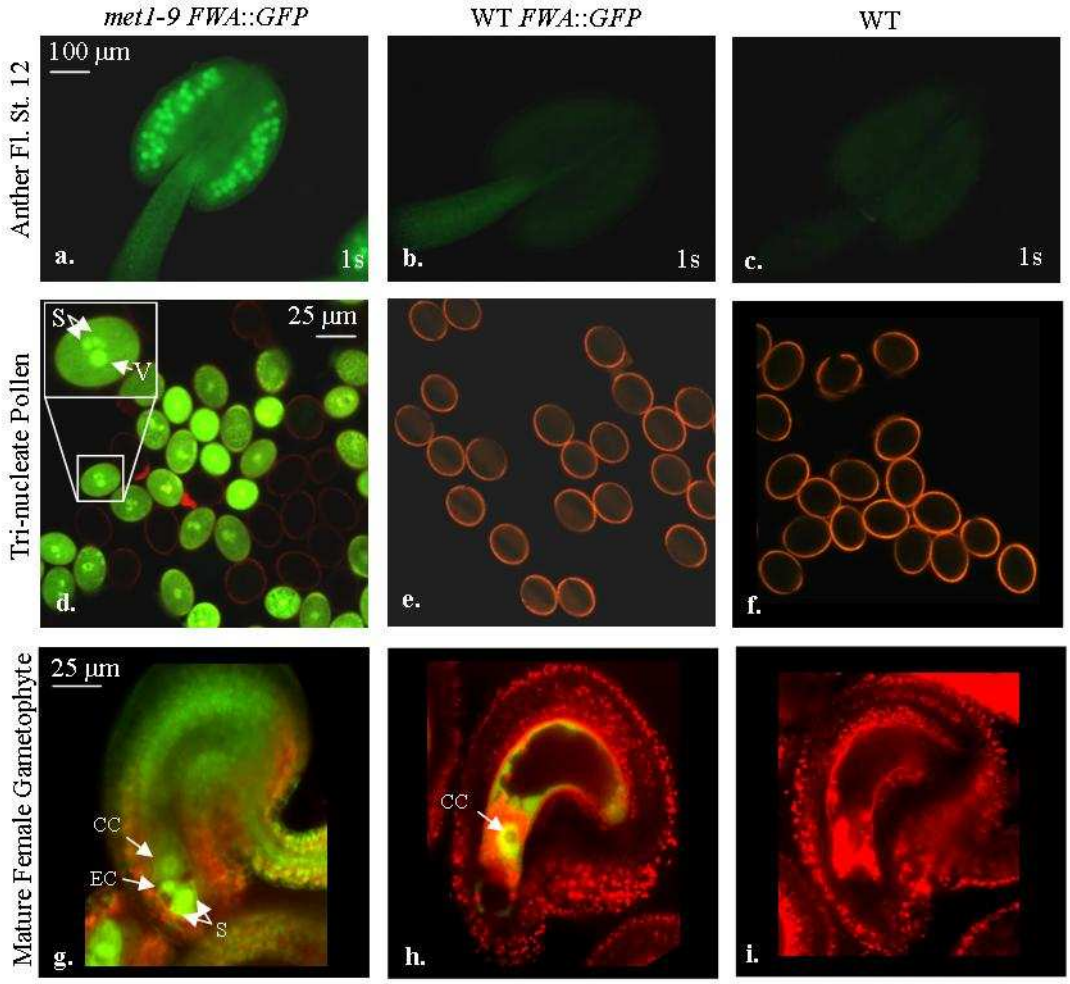


Figure 4.17 *GFP* expression during gametogenesis in WT *FWA*::*GFP* and *met-9 FWA*::*GFP* plants. a., b. and **c.** *GFP* expression in pollen within the anthers at floral stage 12 of *met1-9 FWA*::*GFP,* WT *FWA*::*GFP* and WT plants respectively. The number in the bottom right hand side of images indicates the exposure time of the photographs in seconds. **d., e.** and **f.** *GFP* expression in mature pollen from *met1-9 FWA*::*GFP,* WT *FWA*::*GFP* and WT plants respectively. Arrows point to the vegetative cell nuclei (V) and sperm cell nuclei (S). **g., h.** and **i.** *GFP* expression within female gametophytes of *met1-9 FWA*::*GFP,* WT *FWA*::*GFP* and WT plants respectively. Arrows point to the central cell (CC) and positions of the eggs cell (ES) and synergids (S) are suggested.

4.2.6.2 MEA*::*GUS *expression in WT and* met1-9 *plants*

To assess the ability of the *MEA*::*GUS* reporter to signal hypomethylation, *GUS* activity was analysed in the floral meristem and throughout floral organ development in WT *MEA*::*GUS*[*], *met1-9 MEA*::*GUS*[**] plants. No *GUS* activity was observed in floral meristems or throughout anther, carpel and petal development in *met1-9 MEA*::*GUS* and WT *MEA*::*GUS* plants (**Figure 4.18a** to **f**). Additionally, no *GUS* expression was detected

[*] the *MEA*::*GUS* reporter in a WT background
[**] the *MEA*::*GUS* reporter in a met1-9 homozygous background

in the floral organs of WT plants (**Figure 4.18c** and **f**). However, in both WT *MEA*::*GUS* and *met1-9 MEA*::*GUS* lines, *GUS* activity was observed in pollen of anthers at floral stages 11-12 (**Figure 4.18g and h**). *GUS* activity was also observed in ovules of WT *MEA*::*GUS* plants (**Figure 4.18e).** In contrast, no *MEA* activity was observed in ovules of *met1-9 MEA*::*GUS* plants (**Figure 4.18d**). These results indicate that there is little difference between the expression pattern of the *MEA*::*GUS* reporter in a WT or hypomethylated epigenetic background.

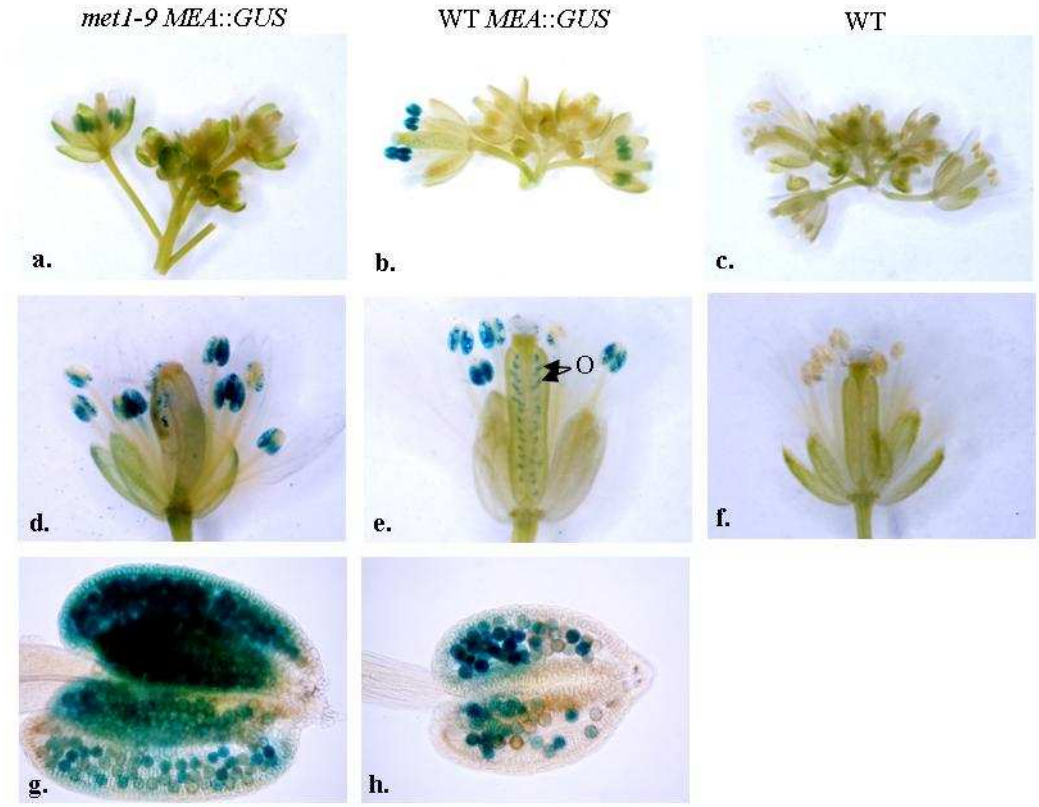


Figure 4.18 *GUS* expression during floral organ development in WT *MEA*::*GUS* and *met1-9 MEA*::*GUS* plants. a., b. and **c.** *GUS* staining in the inflorescence meristem and developing buds of *met1-9 MEA*::*GUS*, WT *MEA*::*GUS* and WT plants respectively. **d., e.** and **f.** *GUS* staining in floral organs at Floral stage 12 from *met1-9 MEA*::*GUS*, WT *MEA*::*GUS* and WT plants respectively. The arrows in **e.** point to GUS staining within ovules (O). **g.** and **h.** *GUS* staining in pollen of *met1-9 MEA*::*GUS* and WT *MEA*::*GUS* plants respectively.

4.2.6.3 mPAC::GFP *expression in WT and* met1-9 *plants*

To assess the ability of the *MEA*::*GUS* reporter to signal hypomethylation, *GFP* expression was analysed in WT *mPAC*::*GFP*[*] and *met1-9 mPAC*::*GFP*[**] plants during anther, carpel

[*] The *mPAC*::*GFP* reporter in a WT back ground

and petal development using fluorescence microscopy. In both WT *mPAC*::*GFP* and *met1-9 mPAC*::*GFP* plants, no fluorescence was observed throughout floral organ development indicating that the *mPAC*::*GFP* reporter is not expressed during this stage of development in either a WT or hypomethylated epigenetic background (**Figure 4.19a, b** and **c**).

GFP expression was also analysed in mature pollen from WT *mPAC*::*GFP* and *met1-9 mPAC*::*GFP* plants using confocal microscopy. Nuclear targeted fluorescence was detected in pollen from WT *mPAC*::*GFP* and *met1-9 mPAC*::*GFP* plants and no fluorescence was detected in WT pollen (**Figure 4.19d, e** and **f**). This finding indicates that there is no difference in the expression of the *mPAC*::*GFP* reporter in pollen from a WT or hypomethylated epigenetic background.

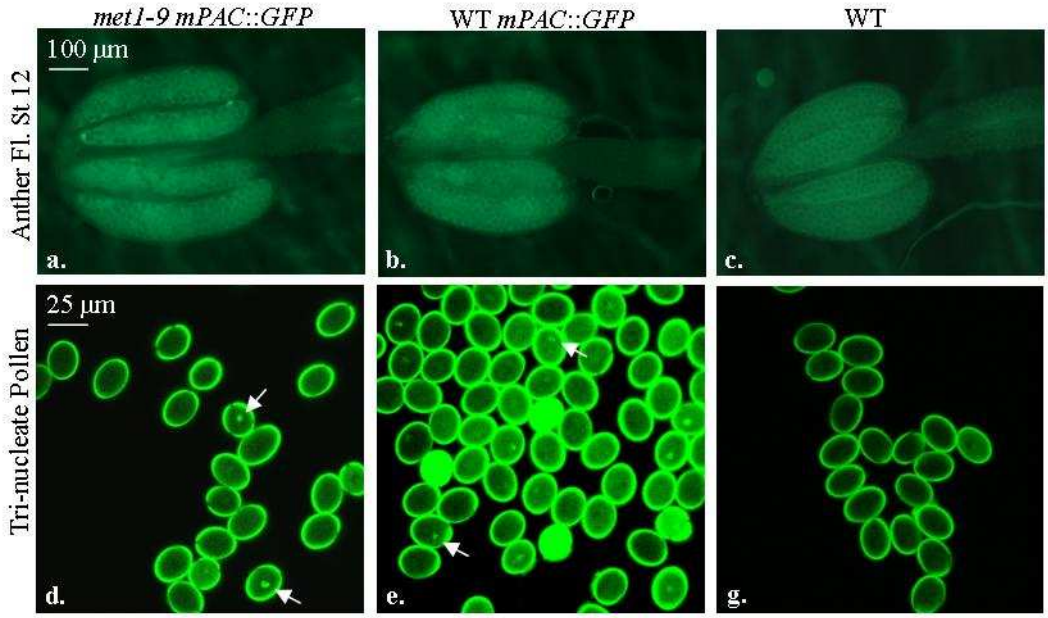


Figure 4.19 *GFP* expression in anther and pollen of *met1-9 mPAC*::*GFP* and WT *mPAC*::*GFP* plants. a., b. and **c.** Fluorescence from anther of *met1-9 mPAC*::*GFP*, WT *mPAC*::*GFP* and WT plants respectively. **d., e.** and **f.** *GFP* expression in nuclei of mature pollen from *met1-9 mPAC*::*GFP*, WT *mPAC*::*GFP* and WT plants respectively. Arrow points to fluorescing nuclei.

4.2.7 *Tools to signal altered genomic imprinting*

The aim of the work described in this section was to identify reporters that can signal when genomic imprinting is altered as a result of parent-of-origin-specific hypomethylation. An

[*] [*] The *mPAC*::*GFP* reporter in a *met1-9* background

appropriate reporter would be imprinted when inherited from a WT background and show loss of parent-of-origin-specific silencing when inherited from hypomethylated epigenetic background. Such criteria are met by the *FWA*::*GFP* reporter. The *FWA*::*GFP* reporter is maternally-expressed and paternally-silenced during endosperm development when inherited from a WT background. However, imprinting is lost and the reporter is biallelically expressed when inherited from a hypomethylated epigenetic background (Chapter 3, **Figure 3.15**). This reporter can therefore be used to signal altered imprinting resulting from parent-of-origin-specific hypomethylation. To test whether the *MEA*::*GUS* and *mPAC*::*GFP* reporters can also be used to signal altered imprinting, the parent-of-origin-specific expression of these reporters was analysed when inherited from a WT and hypomethylated epigenetic background.

4.2.7.1 *Parent-of-origin-specific expression of the* MEA*::*GUS *reporter inherited from a WT and hypomethylated epigenetic background*

GUS activity was analysed in seed produced from reciprocal crosses between WT *MEA*::*GUS* and WT plants at 3DAP. *GUS* expression was detected in [WT *MEA*::*GUS* X WT] seed but not in [WT X WT *MEA*::*GUS*] seed indicating that the *MEA*::*GUS* reporter is imprinted and maternally-expressed/paternally-silenced when inherited from a WT background (**Figure 4.20**). To test whether imprinting of the *MEA*::*GUS* reporter is altered by parent-of-origin-specific hypomethylation, *GUS* activity was analysed in seed produced from reciprocal crosses between *met1-9 MEA*::*GUS* and WT plants at 3DAP. *GUS* activity was detected in [*met1-9 MEA*::*GUS* X WT] seed but not in [WT X *met1-9 MEA*::*GUS*] seed indicting that inheritance of the *MEA*::*GUS* reporter from a hypomethylated epigenetic background does not the disrupt parent-of-origin-specific silencing of this reporter.

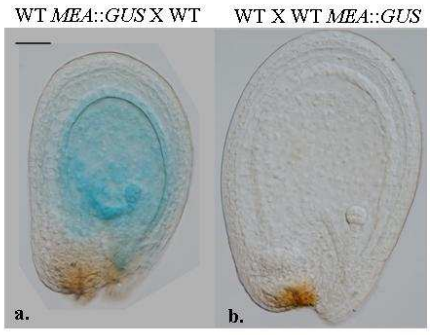


Figure 4.20 Parent-of-origin-specific expression of the *MEA*::*GUS* reporter. GUS activity in [WT *MEA*::*GUS* x WT] (**a.**) and [WT x WT *MEA*::*GUS*] seed (**b.**) at 3DAP. Scale bar 50 μm.

4.2.7.2 *Parent-of-origin-specific expression of the* mPAC*::*GUS *reporter inherited from a WT and hypomethylated epigenetic background*

GFP expression was analysed in seed produced from reciprocal crosses between WT *mPAC*::*GUS* and WT plants at 3DAP using confocal microscopy. *GFP* expression was detected in endosperm nuclei of [WT *mPAC*::*GFP* X WT] seed (**Figure 4.21a**). In contrast, no *GFP* expression was detected in [WT X WT *mPAC*::*GFP*] seed indicating that the *mPAC*::*GFP* reporter is imprinted and maternally-expressed/paternally-silenced when inherited from a WT background (**Figure 4.21b**). To test whether imprinting of the *mPAC*::*GFP* reporter is altered by parent-of-origin-specific hypomethylation, *GFP* expression was analysed in seed produced from reciprocal crosses between *met1-9 mPAC*::*GFP* and WT plants at 3DAP. *GFP* expression was detected in endosperm nuclei of [*met1-9 mPAC*::*GFP* X WT] and [WT X *met1-9 mPAC*::*GFP*] seed (**Figure 4.21c** and **d**). This result indicates that silencing of paternally-inherited *mPAC*::*GFP* is released when inherited from a hypomethylated pollen parent.

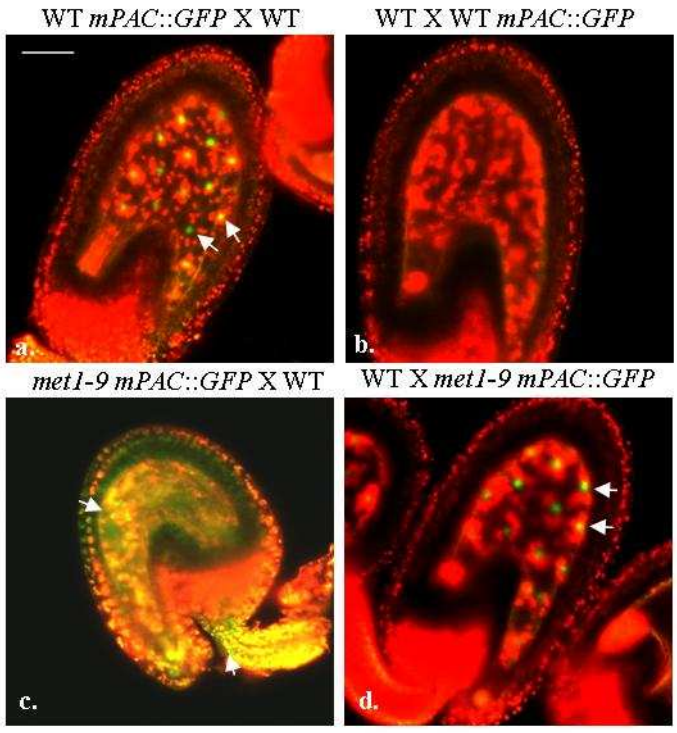


Figure 4.21 Parent-of-origin-specific expression of the mPAC::GFP reporter inherited from a WT and hypomethylated epigenetic background. *GFP* expression in [WT *mPAC*::*GFP* x WT] seed (**a.),** [WT x WT *mPAC*::*GFP*] seed (**b.**), [*met1-9 mPAC*::*GFP* x WT] seed (**c.**) and [WT x *met1-9 mPAC*::*GFP*] seed at 3DAP. Arrows point to examples of nuclear targeted *GFP* expression. Scale bar, 50 μm.

4.3 *Discussion*

The aim of the work described in this chapter was to investigate the expression profile of *MET1* and develop tools to determine when MET1 is required for imprinting.

4.3.1 MET1 *expression during floral organ development and the gametophyte generations*

MET1 expression was analysed during anther and carpel development and the male and female gametophyte generation using the *sMET1*::*GFP* reporter. This section discusses the expression profile of *MET1* and considers the participation of MET1 in imprinting.

On the male side, *sMET1*::*GFP* expression was detected in anther primordia and developing anthers until just prior to flower opening, suggesting that *MET1* is expressed throughout anther development. However, because *GFP* expression was analysed using whole organ fluorescence microscopy, analysis was restricted to the outer cell layers of the anther. For this reason, it cannot be definitively concluded that the *MET1* promoter drives expression in all cells within the anther or in all precursory microspore mother cells, but the seemingly constitutive detection of fluorescence during early anther development is strongly suggestive of this. MET1 is therefore potentially available to maintain methylation at imprinted loci throughout anther development. This is consistent with the predictions of imprinting models 1 and 2 which suggest that MET1 is required to maintain methylation at imprinted loci throughout sporophyte development.

Analysis of *sMET1*::*GFP* expression in microspore mother cells and mono- and bi-nucleate pollen was complicated by nuclear auto-fluorescence. Auto-fluorescence from the microspore wall is a recognised problem for pollen fluorescence microscopy (Johnson-Brousseau, S. A. and McCormick, S. 2004). Additionally, faint nuclear auto-fluorescence is reported in pollen from the conifer *Picea abies* (Lazzaro, M. D. 1999). Despite this problem, the nuclear fluorescence from microspore mother cells and mono- and bi-nucleate pollen of *sMET1*::*GFP* lines was significantly stronger than from WT pollen, suggesting that GFP was present in these cells. It is possible nuclear auto-fluorescence was detected because high sensitivity was required to detect fluorescence from GFP; this infers that fluorescence from GFP may have been very low. A low level of GFP fluorescence could indicate that the reporter was expressed at a low level or that the reporter was not transcribed in the cells under analysis and instead originated from transcripts inherited

from parental cells. In tri-nucleate pollen no auto-fluorescence was detected and GFP was present in two sperm nuclei indicating that *MET1* is expressed in both male gametes. This is in agreement with other studies which have demonstrated a requirement of MET1 during the gametophyte generation (Jullien, P. E. et al. 2006b, Saze, H. et al. 2003, Xiao, W. et al. 2006b, Xiao, W. et al. 2006a).

Imprinting models 1 and 2 predicted that endosperm-promoting genes are demethylated at some point during anther development or the male gametophyte generation, whereas endosperm-promoting genes are demethylation during carpel development or the female gametophyte generation. The apparent low level *MET1* expression in microspore mother cells and mono- and bi-nucleate pollen indicates that genomes may have limited access to MET1 and thus endosperm-promoting genes could become passively demethylation during male gametogenesis. This suggested mechanism of passive demethylation contrasts to the apparently active mechanism of demethylation facilitated by DME, which is appears to de-repress genes during female gametogenesis (Chapter 1, **Figure 1.4**) (Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). However, a reduction in MET1 in microspore mother cells and mono- and bi-nucleate pollen may only result in demethylation of a proportion of gametes; the remainder would inherit the methylation pattern from parental cells (**Figure 4.22**). It is therefore unlikely that passive demethylation resulting from reduced *MET1* expression is alone sufficient to de-repress paternally-inherited imprinted genes

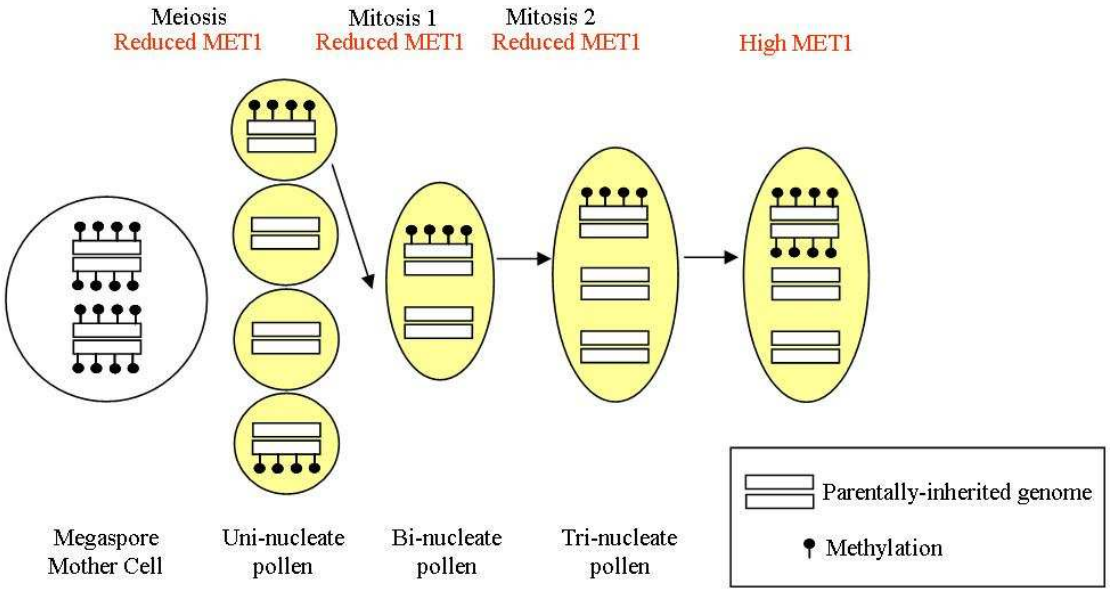


Figure 4.22 Model showing the potential pattern of passive demethylation resulting from reduced MET1 activity during meiosis and the male gametophyte generation. Passive demethylation resulting from reduced MET1 activity during meiosis and in uni- and bi-nucleate pollen results in some tri-nucleate pollen with hemimethylated genomes. MET1 is suggested to methylate the unmethylated stand of hemimethlyated DNA (Saze, H. et al. 2003) therefore this hemimethylated genomes would become fully methylation in response to increase MET1 activity. This model shows that passive demethylation during meiosis and the male gametophyte generation would only demethylate a proportion of gametes male gametes.

On the female side, *sMET1*::*GFP* was expressed in the carpel primordia and in carpels at all stages of floral development indicating that *MET1* is expressed throughout carpel development. Again, because *GFP* expression was analysed using whole organ fluorescence microscopy, the analysis was restricted to the outer cell layers of the carpel. It cannot therefore be definitively concluded that *MET1* is expressed in all cells within the carpel or in all megaspore mother cells precursory cells, but the seemingly constitutive expression of *GFP* is strongly suggestive of this. In support of imprinting models 1 and 2, this indicates that MET1 potentially available to maintain methylation at imprinted loci throughout carpel development

The presence of GFP in the megaspore mother cells and one- two- and four-nucleate gametophyte indicates that *MET1* is expressed during female gametogenesis. Analysis of *GFP* expression in the seven- or eight- nucleate female gametophyte was complicated by fluorescence from the integument cell layers. However, the observation of fluorescence from four or five nuclei (presumably the two synergids, the egg and possibly the pair of

polar nuclei) at the micropylar pole of some embryo sacs strongly suggests that the *MET1* promoter also drives expression in both female gametes. This indicates that MET1 is potentially available to maintain methylation at imprinting loci throughout the female gametogenesis.

4.3.2 MET1 *expression during early seed development*

To investigate the role of MET1 in maintaining DNA methylation-dependent imprints during endosperm development, the *sMET1*::*GFP* reporter was used to analyse the parent-of-origin-specific expression of *MET1* during early seed development. This section discusses the parent-of-origin-specific expression profile of *MET1* and considers the role of MET1 in the maintenance of imprints after fertilization.

Parent-of-origin-specific expression of the s*MET1*::*GFP* reporter was analysed in developing seed from 1-3 DAP. Analysis beyond 3DAP was prevented because by the field depth if the microscope was insufficient to penetrate larger seed. When the *sMET1*::*GFP* reporter was maternally-inherited GFP was detected in proliferating endosperm from 1-3 DAP; in a two nucleate- to a multinucleate-endosperm syncitium. This indicates that *MET1* is maternally expressed throughout early endosperm development.

When the *sMET1*::*GFP* reporter was paternally-inherited, GFP was detected in endosperm at 1DAP, the two- and four-nucleate syncitium, but then the level detected declined such that by 3DAP no GFP was detected in any regions of the endosperm. Explanations for this decline in expression include the possibility that the reporter was transcriptionally silent when paternally-inherited and that the GFP originated from the translation of transcripts inherited from sperm nuclei. In support if this, it is proposed that a large proportion of the paternally-inherited genes are silenced in the endosperm until around 3-4 DAP (Vielle-Calzada, J. P. et al. 2000). This proposal is based on the finding that *GUS* expression from assorted enhancer/gene trap reporter lines was detected during the first cell divisions of the endosperm and/or embryo when maternally-inherited, but not when paternally-inherited (Vielle-Calzada, J. P. et al. 2000). However, exceptions have been found of paternally-inherited genes and transgene which are expressed very shortly after fertilization. For example, in maize transcription of a paternally-inherited 35S::GFP reporter is detected at 4 HAP and translation of the reporter is detected at 6HAP (Scholten, S. et al. 2002).

Additionally, early activation of some paternally inherited loci has been suggested in *Arabidopsis* (Weijers, Dolf et al. 2001). An alternative possibility is therefore that the *sMET1*::*GFP* reporter was expressed immediately after fertilization when paternally-inherited and was subsequently silenced.

When the *sMET1*::*GFP* reporter is inherited either maternally- or paternally-inherited, *GFP* expression in the embryo was detected at 3DAP suggesting that *MET1* is expressed at this stage. Cell division in the embryo is initiated later than in the endosperm. This may explain why GFP expression in the embryo was not detected earlier. As *GFP* expression was detected from the maternally- and paternally-inherited reporter at the same time point, it is suggest that there are no parent-of-origin-specific differences in *MET1* expression during early embryo development.

Imprinting model 1 and 2 predict that that MET1 is required to maintain parent-of-origin-specific methylation of imprinted loci in the endosperm immediately after fertilization until imprinting ceases. Additionally the models predict that MET1 is required to maintain the methylation at imprinted loci in the embryo after fertilization and then throughout sporophyte development. The apparent expression profile of MET1 during early endosperm development is therefore consistent with the predictions of these models. However, the analysis suggests that *MET1* is expressed exclusively from the maternally-inherited allele from at least 2-3 DAP. This indicates that maintenance of methylation at imprinted loci in the endosperm from 2-3 DAP seed is dependent on maternal MET1 i.e. imprinting during early endosperm development is under maternal control. Interestingly, in mammals, the maintenance of DNA-methylation dependent imprinting during the preimplantation development of the embryo is dependent on the expression of the oocyte-specific DNMT1o transcript. Therefore, a maternal-dependence on the maintenance of imprinting during early development appears use the same mechanism in both the plant and animal kingdoms.

4.3.3. *Hypomethylation in* lMET1*::*GFP *lines*

A proportion of *lMET1*::*GFP* plants form a variety of independent transgene insertion lines displayed a *met1*-like phenotype and exhibited ectopic *FWA* expression indicating the transgene can induce hypomethylation. As suggested in section **4.2.3.**, it is possible *lMET1*::*GFP* induces *MET1* co-suppression. In agreement with this hypothesis, semi-

quantitative RT-PCR analysis indicated *met1*-like *lMET1*::*GFP* plants have a lower *MET1* transcript level than a WT control (**Figure 4.8**).

Alternatively, hypomethylation could result from the MET1-GFP translational fusion, encoded by *lMET1*::*GFP,* interfering with MET1 binding to complexes essential for DNA methylation. Yeast two-hybrid and biochemical interaction assays suggest the N-terminal of the mammalian DNA methyltransferase DNMT is capable of binding a number of proteins involved with DNA replication, transcription and chromatin modifications, indicating DNMT1 does not act in an isolated manner (Hermann, A. et al. 2004). For example, DNMT1 binds PROLIFERATING CELL NUCLEARE ANTIGEN (PCNA), an auxiliary factor for DNA replication and repair, (Chuang, L, S. et al. 1997); this interaction facilitates DNMT1 targeting to the replication folk during early and mid S-phase of the cell cycle and enhances methylation efficiency (Schermelleh, L. et al. 2007). Competition assays indicate a peptide derived from the PCNA binding domain of DNMT1 disrupts DNMT1-PCNA binding (Chuang, L, S. et al. 1997). MET1-GFP could compete with MET1 and disrupt similar protein-protein interactions involved in plant DNA methylation.

4.3.4 *Tools to investigate when MET1 is required for genomic imprinting*

As discussed in Chapter 1 (section **1.4.2.6.**), the approach chosen to test when MET1 is required for imprinting was to suppress *MET1* tissue-specifically and assay for altered imprinting. In preparation for this, the aim of the work described in this chapter was to develop an approach to suppress *MET1* and tools to signal tissue-specific hypomethylation and altered genomic imprinting.

4.3.4.1 MET1 *suppression* ***by RNAi-induced PTGS***

To investigate when MET1 is required for imprinting using the approach described above, it was necessary to develop an effective and reliable mechanism to suppress *MET1* sufficiently to induce hypomethylation and loss of imprinting. *MET1* suppression by RNAi-induced PTGS was accordingly investigated by analysing ChromDB *MET1*-RNAi were and novel *35S*::*MET1*-RNAi lines for hypomethylation and loss of imprinting.

The *MET1* transcript level in ChromDB *MET1*-RNAi lines was found to be equivalent to that in WT plants and raised the possibility that *MET1* was immune to RNAi-induced-PTGS (**4.2.4.1**). Work on these lines was stopped. However, a substantially reduced *MET1*

transcript level and ectopic expression of *FWA* was evident in novel *35S*::*MET1*-RNAi lines whose design and construction is described here. This indicates that *MET1* can be suppressed by RNAi-induced-PTGS. Possible co-suppression of *MET1* by *lMET1*::*GFP* could provide additional evidence, as the co-suppression pathway is reportedly analogous to the RNAi pathway (Hamilton, A. J. and Baulcombe, D. C. 1999).

The reduced *MET1* transcript level and ectopic *FWA* expression in *35S*::*MET1*-RNAi lines also indicate that the *35S*::*MET1*-RNAi transgene effectively induces *MET1* suppression and hypomethylation. The *met1*-like phenotype of *35S*::*MET1*-RNAi lines provides additional evidence of this. Moreover, the reciprocal parent-of-origin-specific effects of seed weight resulting from the inheritance of genomes from *35S*::*MET1*-RNAi lines indicates that the level of hypomethylation induced in these lines was sufficient to release silencing of endosperm-promoting and –inhibiting genes. The ability of pollen from *35S*::*MET1*-RNAi lines to rescue seed abortion of *mea* mutants provides yet more evidence that the *35S*::*MET1*-RNAi is highly effective. In conclusion, the *MET1*-RNAi transgene is an effective tool to suppression MET1 and induce hypomethylation and altered imprinting. It is therefore suggested that this transgene will be effective in suppressing *MET1* tissue-specifically hypomethylation when driven from tissue-specific promoters.

4.3.4.2 *Tools to signal hypomethylation*

As exemplified by the *35S*::*MET1*-RNAi and the *met1-9* lines, constitutive suppression of *MET1* causes phenotypic abnormalities which are easily detectable and diagnostic of hypomethylation. In contrast, tissue-specific suppression of *MET1* may not cause any obvious phenotypic abnormalities making it difficult to verify tissue-specific suppression of *MET1*. With the aim of identifying a tool to signal tissue-specific hypomethylation, the expression of a number of imprinted gene promoter-reporter constructs was compared in a WT and hypomethylated epigenetic background during floral organ development.

The *MEA*::*GUS* reporter had a similar expression pattern in WT and *met1-9* hypomethylated plants. No GUS activity was detected during anther and carpel development in either epigenetic background indicating that silencing of the *MEA*::*GUS* reporter is not repressed by hypomethylation in these tissues. This is consistent with the recent finding that the sporophytic silencing of *MEA* is maintained by histone methylation rather than DNA methylation (Jullien, P. E. et al. 2006a). However, GUS activity was

detected in pollen from both WT and *met1-9* plants. This was surprising because MEA is reportedly not expressed in pollen (Choi, Y. et al. 2004). It is therefore apparent that the *MEA*::*GUS* reporter does not signal *MEA* expression accurately in during male gametogenesis.

The *MEA*::*GUS* reporter did show a different expression pattern in ovules from *met1-9* and WT plants. GUS activity was present in ovules from WT *MEA*::*GUS* plants but not *met1-9 MEA*::*GUS* plants. *met1-9* plants reportedly have severely reduced fertility as seed parents (Chapter 3, section **3.2.5.2**). It is possible that absence of *GUS* expression in *met1-9 MEA*::*GUS* ovules is caused by altered ovule development as opposed to hypomethylation of the ovule *per se*. Altered ovule development is likely to result from hypomethylation in ovule precursory tissue and therefore absence of *MEA*::*GUS* expression is unlikely to be a reliable signal for tissue-specific hypomethylation in the ovule alone. As the *MEA*::*GUS* reporter has a very similar expression pattern in WT and hypomethylated *met1-9* plants, this reporter is not a useful tool to signal tissue-specific hypomethylation.

The *mPAC*::*GFP* reporter has a similar expression pattern in WT and *met1-9* hypomethylated homozygous background. In both WT *mPAC*::*GFP* and *met1-9 mPAC*::*GFP* plants *GFP* was not expressed during anther or carpel development but GFP was present in pollen. This is surprising because preliminary RT PCR analysis indicated that native *mPAC* transcript is not present in pollen (Sushma Tiwari, Personal Communication). As with *MEA*::*GUS* reporter, it is likely that the *mPAC*::*GFP* reporter fails to signal expression accurately in pollen. Activation of these reporters in pollen could reflect a phase of reduced DNA methyltransferase activity in pollen as suggested from the *MET1* expression analysis. Alternatively, it could reflect a phase of chromatin remodelling or epigenetic reprogramming during gametogenesis. Nevertheless, the *mPAC*::*GFP* reporter is not a useful tool to signal hypomethylation.

In contrast to the preceding genes, the *FWA*::*GFP* reporter has a very different expression pattern in WT and *met1-9* hypomethylated plants. During anther and carpel development strong *GFP* expression was detectable in *met1-9* plants compared to weak expression in WT plants. This indicates that the reporter is capable of signally hypomethylation during floral organ development. Additionally, GFP was present in both pollen and multiple nuclei of female gametophytes from *met1-9 FWA*::*GFP* plants, but absent in pollen from

WT *FWA*::*GFP* plants. As expected, GFP was restricted to the central cell of female gametophytes from these plants. This indicates that the *FWA*::*GFP* reporter can also signal hypomethylation during gametogenesis.

One disadvantage with the *FWA*::*GFP* reporter is that it is weakly expressed during anther and carpel development in a WT background indicating that the reporter may be unable to signal low levels of hypomethylation. Additionally background reporter expression could be misinterpreted as hypomethylation. To avoid this problem it is necessary to compare test tissue with WT *FWA*::*GFP* tissue directly and to interpret *GFP* expression as hypomethylation only when the signal from the test tissue is significantly stronger than that from WT *FWA*::*GFP* plants. Overall it is concluded that *FWA*::*GFP* reporter is likely to be an effective tool for signalling tissue-specific hypomethylation.

4.3.4.3 *Tools to signal altered genomic imprinting*

With the aim of identifying reporters to signal altered genomic imprinting as a result of tissue-specific hypomethylation, the parent-of-origin specific expression of *MEA*::*GUS* and *mPAC*::*GFP* was analysed in developing endosperm when inherited from a WT and *met1-9* hypomethylated background. An appropriate reporter would be imprinted when inherited from a WT background and show loss of parent-of-origin-specific silencing when inherited from a hypomethylated background.

The *MEA*::*GUS* reporter was maternally-expressed and paternally-silenced when inherited from either a WT or *met1-9* hypomethylated background. This is in agreement with the recent findings that the paternal silencing of MEA during gametogenesis and early endosperm development is controlled by histone methylation and not DNA methylation (Gehring, M. et al. 2006, Jullien, P. E. et al. 2006a). For these reasons, the *MEA*::*GUS* reporter is not a useful tool to signal altered genomic imprinted a resulting from hypomethylation.

The *mPAC*::*GFP* reporter is maternally-expressed and paternally-silenced when inherited from a WT background and biallelically-expressed when inherited from *met1-9* hypomethylated background. These results indicate that the transcriptional silencing of the *FWA*::*GFP* transgene to be paternally-inherited reporter is dependent on MET1 activity. The *mPAC*::*GFP* reporter has the same parent-of-origin-specific expression pattern as the

FWA::*GFP* reporter when inherited from WT and *met1-9* hypomethylated background (Chapter 3, section **3.2.6.3**). It is concluded that both the *mPAC*::*GFP* and the *FWA*::*GFP* reporter are useful tools to signal altered genomic imprinted resulting from hypomethylation of the paternally-inherited genome.

Having constructed a MET1-RNAi transgene capable of suppressing *MET1* and tools to signal hypomethylation and altered imprinting, further work can now focus on using these tools to suppress MET1 tissue-specific and to subsequently analyses the effects on methylation and imprinting. This is described in Chapter 5.

Chapter 5 - *A transgenic approach to suppress MET1 tissue-specifically*

5.1 *Introduction*

In Chapter 1 (section **1.4.2.5**), two models of DNA methylation-dependent imprinting were proposed which differ in their requirement for MET1 during the gametophyte generation. Briefly, both models require that the default state of imprinted genes is methylated and silent and that imprinting is achieved by sex-specific demethylation. Additionally, both models require MET1 throughout sporophytic development to maintain methylation at imprinted loci. Model 1 also requires MET1 throughout the gametophyte generation to maintain methylation of the silenced allele (Chapter 1, **Figure 1.5**). In contrast, Model 2 assumes that imprinted loci are sex-specifically 'marked' prior to sporogenesis by an epigenetic modification which ensures that they are silent in the endosperm. This 'mark' is maintained during the gametophyte generation independently of MET1 and hence Model 2 does not require MET1 to participate in imprinting during the gametophyte generation (Chapter 1, **Figure 1.6**).

As a consequence of their tissue-specific requirements for MET1, models 1 and 2 predict mutually-distinct effects on imprinting resulting from tissue-specific MET1 suppression. Models 1 and 2 predict that *MET1* suppression during anther and carpel development will release default silencing of endosperm-inhibiting and endosperm-promoting genes respectively. Model 1 predicts the same effects will result from *MET1* suppression during the male and female gametophyte generation. In contrast, Model 2 predicts that *MET1* suppression during the male and female gametophyte generation will have no effect on imprinting. The validity of these models can therefore be assessed by determining when MET1 is required for imprinted by suppressing *MET1* tissue-specifically and testing for altered imprinting.

Models 1 and 2 also predict that tissue-specific *MET1* suppression will alter seed size (Chapter 1, **1.4.2.7**). Reportedly, released silencing of paternally-inherited endosperm-inhibiting genes restricts endosperm proliferation and reduces seed weight, whereas released silencing of maternally-inherited endosperm-promoting genes promotes endosperm proliferation and increases seed weight (Adams, S. et al. 2000). According to Models 1 and 2, the progeny of lines suppressing *MET1* during anther development should therefore be epigenetically and phenotypically similar to [WT x *MET1as*] seed and weigh

less than WT seed. In contrast, the progeny of lines suppressing *MET1* during carpel development should be epigenetically and phenotypically similar to [*MET1as* x WT] seed and weigh more than WT seed. Consequently, tissue-specific *MET1* suppression could be used to engineer seed size.

The aim of the work described in this chapter was to investigate whether a transgenic approach is effective in suppressing *MET1* tissue-specifically. This approach could be used to determine when *MET1* is required for imprinting and to develop a practical approach to engineer seed size. Chapter 4 described the development of a transgene that successfully induced DNA hypomethylation and altered imprinting when expressed from the CaMV 35S promoter. This chapter describes the development of derivatives of this transgene designed to suppress *MET1* tissue-specifically.

5.2 *Results*

5.2.1 *The production of promoter-specific* MET1-*RNAi lines*

Chapter 4 described the development of a 35s::*MET1*-RNAi transgene which triggers RNAi-induced PTGS of *MET1* and the hypomethylation of imprinting loci. To suppress *MET1* during only anther or carpel development, or male or female gametogenesis, lines carrying the *MET1*-RNAi transgene driven from tissue-specific promoters were produced. This section describes the production and preliminary analysis of these lines.

5.2.1.1 *Choice of tissue-specific promoters*

A literature search was carried out to identify potentially appropriate tissue-specific promoters to drive *MET1*-RNAi expression.

The promoter region of *APETALA3* (*AP3*) was chosen as a carpel-specific promoter. *AP3* encodes a MADS box-containing protein and is a B class floral organ identity gene which functions to promote petal and stamen identity. *In situ* hybridization analysis suggests that *AP3* is expressed in petals and stamen precursory cells of the floral meristem shortly after Floral stage 3, in petal and stamen primordia and throughout petal and stamen development until at least Floral stage 10 (Jack, T. et al. 1992). No *AP3* transcript is detected in vegetative or root tissue suggesting that *AP3* expression is petal- and stamen-specific. A construct carrying an *AP3* promoter fragment, consisting of 1.7 kb 5' to the *AP3* transcriptional start (GenBank accession U30729) driving *AP3* cDNA, was shown to

completely rescue the *ap3* mutant phenotype (Irish, V. F. and Yamamoto, Y. T. 1995). This promoter fragment therefore includes all the spatial and temporal regulatory motifs necessary to drive the tissue-specific expression.

In accordance with the expression profile of AP3, the *AP3* promoter was expected to drive *MET1*-RNAi expression and thus suppress *MET1* expression and induce hypomethylation during anther and petal development from floral stage 3-10. The *Arabidopsis* genome is reportedly slow to recover from hypomethylation (Finnegan, E. J. et al. 1996, Genger, R. K et al. 1999, Kakutani, T. et al. 1999), therefore it was predicted that anthers would remain hypomethylated beyond Floral stage 10 and that genomes paternally-inherited from *AP3*::*MET1*-RNAi lines would remain hypomethylated during seed development.

The promoter region of *SHATTERPROOF2* (*SHP2*) was chosen as a carpel-specific promoter. *SHP2,* also known as *AG-LIKE 5* (*AG5*), is a MADS box gene that plays a role in promoting carpel and ovule identity and fruit dehiscence (Liljegren, S. J. et al. 2000, Ma, H. et al. 1991, Pinyopich, A. et al. 2003). The expression of SHP2 is regulated by the floral identity gene *AGAMOUS* (*AG*). AG binds to the *SHP2* promoter and can induce ectopic expression of AG activates ectopic SHP2 expression (Savidge, B. et al. 1995). *In situ* hybridization analysis indicates that *SHP2* is expressed uniformly in developing carpels from Floral stage 6-8. Subsequently, *SHP2* expression is restricted to ovule primordia, the septum and a region of the ovary wall and, as ovules differentiate expression, becomes restricted to the inner integument and funiculus (Savidge, B. et al. 1995).

The *SHP2* promoter fragment chosen to drive the *MET1*-RNAi construct consists of 1.5 kb 5' to the *SHP2* transcriptional start. This region includes the AG binding site identified by Savidge et al (1995). The *SHP2*::*MET1*-RNAi was expected to induce hypomethylation during carpel development from Floral stage 6-8. Carpels were predicted to remain hypomethylated beyond Floral stage 8 and genomes maternally-inherited from *SHP2*::*MET1*-RNAi lines were predicted to remain hypomethylated during seed development.

The promoter region of *APG* was chosen as a male gametophyte-specific promoter. *APG* was initially identified by probing the *Arabidopsis* genomic library with a microspore

specific cDNA identified in tobacco (Roberts, M. R. et al. 1991, Roberts, M. R. et al. 1993). A *APG*::*GUS* reporter construct, consisting of 530 bp 5' and 11 bp 3' to the APG transcriptional start fused to *GUS*, showed anther-specific expression in *Arabidopsis* and tobacco (Roberts, M. R. et al. 1993). Further analysis in tobacco showed that the reporter is not expressed prior to or during meiosis and reporter expression is restricted to mononuclear microspores and during microspore mitosis (Roberts, M. R. et al. 1993). Expression was also reported in the tapetum, the stomium and the anther wall.

The same *APG* promoter fragment as described by Roberts et al (1993) was chosen to drive the *MET1*-RNAi construct. This was expected to drive *MET1*-RNAi expression and thus induce hypomethylation during the male gametophyte generation. Genomes paternally-inherited from *APG*::*MET1*-RNAi lines were predicted to remain hypomethylated during seed development.

The promoter region of *At2g20070* was chosen as a female gametophyte-specific promoter. *At2g20070* is a female gametophyte-specific gene identified from a transcriptome comparison of ovules from WT and *sporocytless* mutants, which produces ovules with no female gametophyte (Yu, H. et al. 2005). *At2g20070* transcript was detected at high levels in WT ovules and at very low levels in *sporocytless* ovules. Female gametophyte specific expression of *At2g20070* was confirmed by the expression of an *At2g20070::GUS* reporter, consisting of a 440 bp 5' to *At2g20070* fused to *GUS* (Yu, H. et al. 2005). Expression of the reporter was detected during division of the female gametophyte from FG1-GF3 and at the chalazal end of the female gametophyte at FG4. Expression of the *At2g20070::GUS* reporter was not detected in other floral organs or in vegetative tissue.

The same *At2g20070* promoter fragment described by Yu et al (2005) was chosen to drive the *MET1*-RNAi construct. This was expected to induce hypomethylation during the female gametophyte generation and genomes maternally-inherited from *At2g20070*::*MET1*-RNAi lines were predicted to remain hypomethylated during seed development..

5.2.1.2 *Construction of* AP3*::*MET1-*RNAi,* SHP2*::*MET1-*RNAi,* APG*::*MET1-*RNAi and* At2g20070*::*MET1-*RNAi constructs*

The *AP3*, *SHP2*, *APG* and *At2g20070* promoter fragments were amplified from WT Col-0 DNA by PCR using primers AP3F & AP3R, SHP2F & SHP2R, APGF & APGR and At2g20070F & At2g20070R respectively. *AP3*, *SHP2*, *APG* and *At2g20070* promoter PCR fragments were ligated into pGEM-T to produce *AP3*::*pGEMT*, *SHP2*::*pGEMT, APG*::*pGEMT* and *At2g20070*::*pGEMT* (**Figure 5.1a**). Following transformation into *E. coli,* positive colonies for each construct were sequenced from the 3' end of the promoter fragment. No errors were detected from the sequence reads. Each promoter fragment was cut from *pGEMT* with *Eco*RI and *Nco*I. Additionally, the CaMV *35S* promoter was cut from the *35S*::*MET1*-RNAi construct using the same enzymes. The promoter fragments were then ligated into the *Eco*RI- and *Nco*I-cut *MET1*-RNAi backbone to produce *AP3*::*MET1*-RNAi, *SHP2*::*MET1*-RNAi, *SHP2*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi constructs (**Figure 5.1b**).

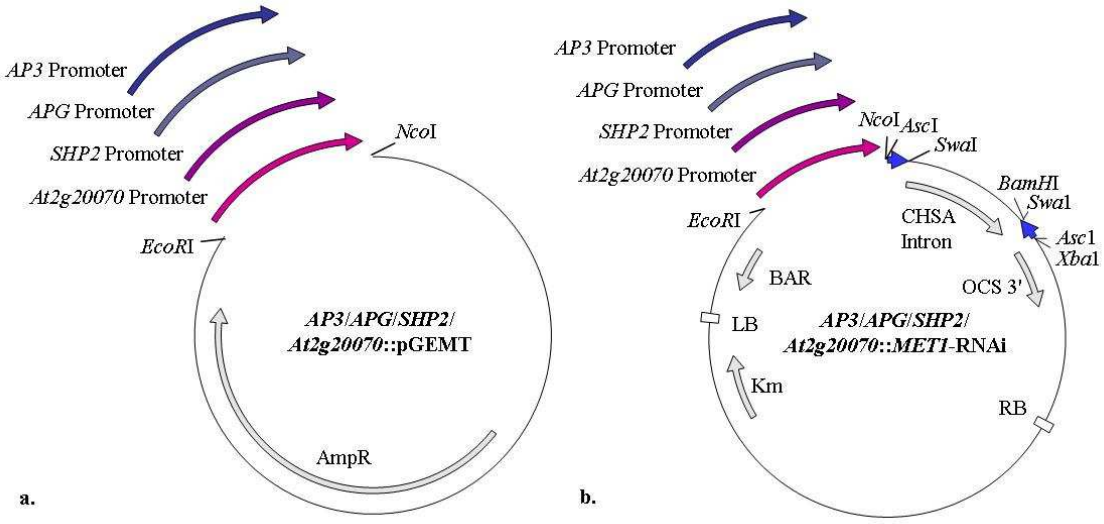


Figure 5.1 Plasmids maps of *AP3*/*SHP2*/*APG*/*At2g20070*::*pGEMT* (a) and *AP3*/*SHP2*/*APG*/*At2g20070*::*MET1*-RNAi (b).

5.2.1.3 *Transformation of the promoter-specific* MET1-*RNAi transgenes into* Arabidopsis

AP3::*MET1*-RNAi, *SHP2*::*MET1*-RNAi, *APG*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi constructs were transformed into *A. tumefaciens* and then the transgenes were transformed into the *Arabidopsis* accession Col-0 by floral dipping. T1 seedlings were treated with BASTA to select for transformants. 10 T1 *AP3*::*MET1*-RNAi plants, 9 T1 *SHP2*::*MET1*-

RNAi plants, 19 T1 *APG*::*MET1*-RNAi plants and 32 *At2g20070*::*MET1*-RNAi plants were identified. PCR was performed on BASTA-resistant plants to check for the presence of the transgene. Transgenes were detected in all plants tested.

5.2.1.4 *Selection of promoter-specific* MET1-*RNAi lines*

Due to time restrictions only a sample of promoter-specific *MET1*-RNAi lines were selected for further analysis. Models 1 and 2 predict that tissue-specific *MET1* suppression will affect seed weight; predicted trends in the weight of seed produced from promoter-specific *MET1*-RNAi lines are summarised in **Table 5.1**. With respect to these predictions, the choice of promoter-specific RNAi lines to use for analysis was based on the weight of seed produced from T1 lines.

Table 5.1 Seed weight trends of promoter-specific *MET1*-RNAi lines predicted by imprinting models 1 and 2

Line	Model 1	Model 2
AP3::*MET1*-RNAi	Less than WT	Less than WT
SHP2::*MET1*-RNAi	More than WT	More than WT
APG::*MET1*-RNAi	Less than WT	Equal to WT
At2g20070::*MET1*-RNAi	More than WT	Equal to WT

The mean T2 seed weight from each promoter-specific line was compared with the mean weight of WT seed (**Figure 5.2**). One-way ANOVAs identified significant differences in the seed weighs of lines carrying the same promoter-specific MET1-RNAi transgene and WT plants (**Table 5.2**). Post-hoc Dunnett's tests identified lines with seed weights which significantly differed from the WT control (**Table 5.2**). Lines with seed weigh which differed from WT in the predicted direction were selected for further analysis.

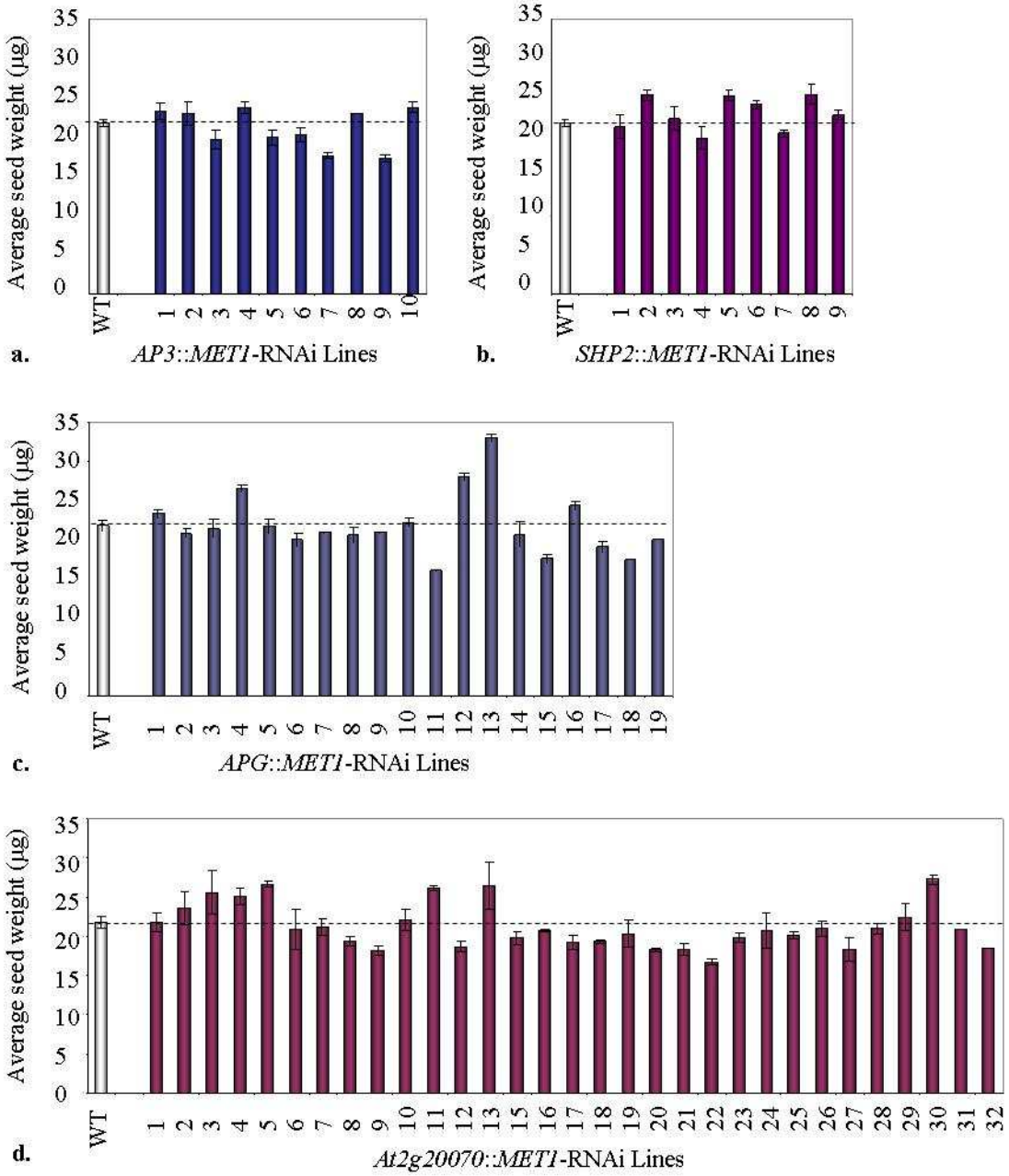


Figure 5.2 T2 seed weights from promoter-specific *MET1*-RNAi lines. The average seed weight is the mean of the average seed weight calculated for three to five pods per plant. Error bars show standard error. The dotted line across each graph shows the average WT seed weight.

Table 5.2 Summary of statistical analyses comparing T2 seed weigh from promoter-specific MET1-RNAi lines		
Comparison	One-way ANOVA	Dunnett's test[a]
WT vs. *AP3*::*MET1*-RNAi lines	$F_{10,41}$=5.41, P=0.000	AP3 L7 (7.761,-0.485) AP3 L9 (8.153,-0.877)
WT vs. *APG::MET1*-RNAi lines	$F_{19,61}$=19.20, P=0.000	APG L4 (2.245,7.258) APG L11 (-8.848,-2.605) APG L12 (3.146,9.396) APG L13 (8.140,14.383) APG L15 (-7.389, -1.146) APG L18 (-7.498,-1.255)
WT vs. *SHP2::MET1*-RNAi lines	$F_{8,37}$=3.33, P=0.006	SHP9 L2 (0.269,7.040)
WT vs. *At2g20070::MET1*-RNAi lines	$F_{31,85}$=4.13, P=0.000	At2g20070 L5 (0.498,9.284 At2g20070 L30 (0.113,10.842)
[a] Lines which have a mean seed weight which significantly differs from the mean seed weight of the WT control plants. 95% confidence interval for the difference between the mean of each test lines and the mean of the WT control shown in brackets. Grey highlights lines which significantly differ from WT in the direction predicted (See **Table 5.1**)		

5.2.2 *Imprinting analysis of promoter-specific* MET1-*RNAi lines*

This section describes the results from three approaches that were used to test for release-silencing of imprinted genes paternally-inherited from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines and maternally-imprinted from *SHP2*::*MET1*-RNAi lines and *At2g20070*::*MET1*-RNAi lines.

5.2.2.1 *Seed weight analysis of promoter-specific* MET1-*RNAi lines*

Loss of imprinting in promoter-specific *MET1*-RNAi lines was first evaluates by comparing the weight of seed produced from these lines with that of WT plants. The seed analysed was collected from T2 plants which had been subjected to restricted seed set. **Figure 5.3** shows the ratio of the weight of T3 seed from each promoter-specific *MET1*-RNAi line to that from WT plants.

The weight of seed produced from *AP3*::*MET1*-RNAi and *APG*::*MET*1-RNAi lines was equal to or slightly heavier than that from WT plants. These results suggest silencing is not released from endosperm-inhibiting genes that are paternally-inherited from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines.

The weight seed produced from a number of *SHP2*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi lines was greater than that from WT plants whereas the weight of seed of other lines was less. These findings suggest it is unlikely that silencing is released from endosperm-

promoting genes that are maternally-inherited from these lines. It should also be noted that *SHP2*::*MET1*-RNAi line 6, which produced considerably heavier seed than WT plants, had reduced fertility and produced approximately half the number of seeds per pod compared to WT plants. In *Arabidopsis* increased seed weight is positively-correlated with a reduction in the number of seed set per pod (Adams, S. et al. 2000), therefore it is likely that increased seed weight of *SHP2*::*MET1*-RNAi line 6 was caused by reduced fertility as opposed to the released-silencing of maternally-inherited endosperm-promoting genes.

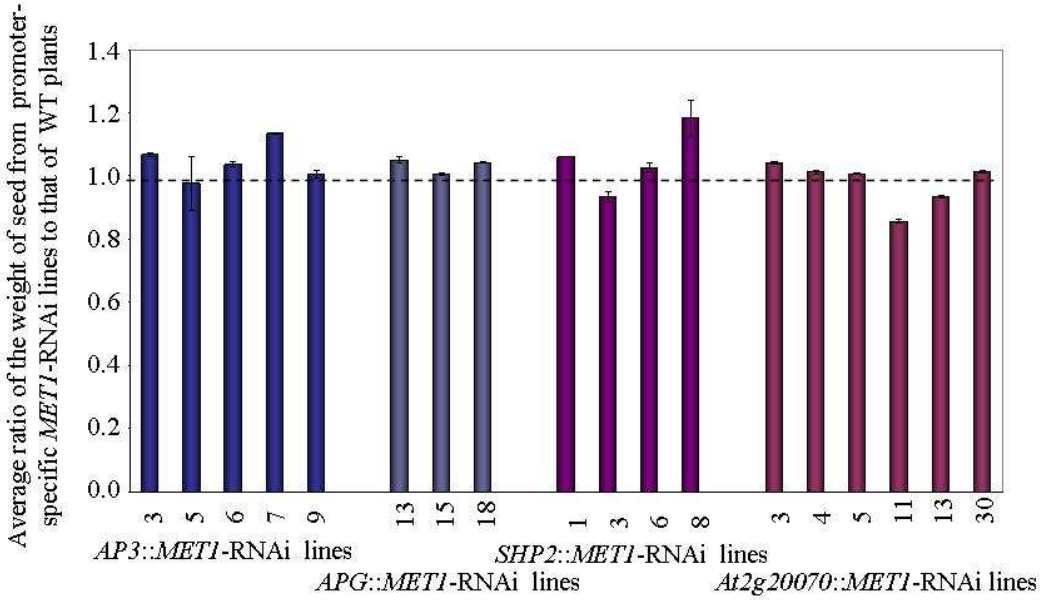


Figure 5.3 Seed weight analysis of promoter-specific *MET1*-RNAi lines. The average seed weight for five plants per line was calculated by determining the mean weight of seed from six pods per plant. The ratio of the seed weight from each promoter-specific *MET1*-RNAi line to that of WT plants was calculated for five plants per lines. This graph shows the average ratio of the seed weight of each promoter-specific *MET1*-RNAi line to that from WT. Error bars show the standard error.

5.2.2.2 *The ability of pollen from* AP3*::*MET1-*RNAi and* APG*::*MET1-*RNAi lines to rescue seed abortion of* mea *mutants*

The second approach to evaluate *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines for loss of imprinting was to analyse the ability of pollen to rescue seed abortion of *mea* mutants. Seed abortion of *mea* mutants is associated with endosperm over-proliferation and rescue of abortion by hypomethylated pollen is likely due to released silencing of paternally-inherited endosperm-inhibiting genes (Chapter 1, **1.4.2.1**). Rescue of seed abortion by

AP3::*MET1*-RNAi and *APG*::*MET1*-RNAi pollen would therefore indicate the imprinting of endosperm-inhibiting genes is disrupted in these lines.

mea mutants were emasculated and pollinated by *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines and the number of shrivelled vs plump seed per pod was scored at 14 DAP. The level of seed abortion from crosses between *mea* mutants and *AP3*::*MET1*-RNAi or *APG*::*MET1*-RNAi lines was comparable to that from crosses between *mea* mutants and WT plants (**Table 5.3**). These results further suggest that silencing is not released from endosperm-inhibiting genes that are paternally-inherited from these lines. However, as suggested in section **3.2.6.2.**, the La-er/Col-0 hybrid background created by these crosses could affect the ability to rescue seed abortion. To rule this possibility out these crosses should be repeated using plants with the same genetic background.

Table 5.3 Percentage seed abortion from crosses between *mea* mutants and *AP3*::*MET* –RNAi or *APG*::*MET* –RNAi lines.

Cross [a]	Mean % Seed Abortion [b]	S.E.M. [c]	Total number of seed analysed
mea (La-*er*) X *AP3*::*MET1*-RNAi line 7 (Col-0)	96.4	0.2	793
mea (La-*er*) X *AP3*::*MET1*-RNAi line 9 (Col-0)	95.9	0.8	543
mea (La-*er*) X WT (Col-0)	93.9	0.3	346
mea (La-*er*) X *APG*::*MET1*-RNAi line 11 (Col-0)	97.4	0.7	217
mea (La-*er*) X *APG*::*MET1*-RNAi line 15 (Col-0)	95.4	1.8	239
mea (La-*er*) X *APG*::*MET1*-RNAi line 18 (Col-0)	95.1	1.3	297
mea (La-*er*) X WT (Col-0)	94.4	2.9	95

[a] The genetic background of the plants shown in brackets
[bc] Calculated from the percentage seed abortion in three to five pod from two to three pollen parents per line.

5.2.2.3 *Parent-of-origin-specific expression of the* FWA*::*GFP *reporter inherited from* AP3*::*MET1-*RNAi and* APG*::*MET1-*RNAi lines*

The *FWA*::*GFP* reporter was used to further test for released-silencing of imprinted genes paternally-inherited from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines. In Chapter 3, (section **3.3.6.3**) it was demonstrated that the *FWA*::*GFP* reporter is imprinted and expressed only when maternally-inherited. However, when inherited from a hypomethylated *met1-9* background, imprinting is lost and the reporter is expressed when maternally- *and* paternally-inherited. Expression of *FWA*::*GFP* reporter when paternally-inherited from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines would therefore show that these lines lack paternal imprinting.

AP3::*MET1*-RNAi lines 7 and 9 and *APG*::*MET1*-RNAi lines 11, 15 and 18 were crossed with WT *FWA*::*GFP* lines. F1 plants hemizygous for the promoter-specific *MET1*-RNAi transgene and the reporter were reciprocally crossed with WT plants and 3DAP seed was analysed for *GFP* expression using fluorescence and confocal microscopy. When the *AP3*::*MET1*-RNAi *FWA*::*GFP* and *APG*::*MET1*-RNAi *FWA*::*GFP* lines were used as seed parents, *GFP* expression was detected in 50 % of seed; this is consistent with segregation of the *FWA*::*GFP* reporter into 50 % of gametes. However, when the lines were used as pollen parents no *GFP* expression was detected. In total, approximately 900 seeds from each cross were screened. These results further suggest that silencing is not released from imprinted loci paternally-inherited from these lines.

5.2.3 *Methylation analysis of promoter specific MET1::RNAi lines*

Promoter-specific expression of the *MET1*::RNAi construct was predicted to induce tissue-specific hypomethylation and altered imprinting by releasing gene-silencing. However, the analysis described above suggests that silencing is not released from imprinted loci in any lines tested. These results could indicate that tissue-specific hypomethylation has no effect on imprinting. Alternatively, it is possible that promoter-specific *MET1*::RNAi transgenes do not induce tissue-specific hypomethylation. To test this, the *FWA*::*GFP* reporter was used to assay for tissue-specific hypomethylation of the promoter-specific *MET1*::RNAi lines.

Promoter-specific *MET1*::RNAi lines were crossed with WT *FWA*::*GFP* lines to produce promoter-specific *MET1*-RNAi *FWA*::*GFP* lines and these lines were analysed for ectopic *FWA*::*GFP* expression. In Chapter 4 (section **4.2.6.1**), it was demonstrated that the *FWA*::*GFP* reporter is ectopically expressed during anther and carpel development and the male and female gametophyte generation in a *met1-9* hypomethylated epigenetic background. Ectopic expression of the *FWA*::*GFP* reporter in promoter-specific *MET1*-RNAi *FWA*::*GFP* lines would therefore indicate that hypomethylation has been induced.

5.2.3.1 *Methylation analysis of* AP3*::*MET1-*RNAi lines*

AP3::*MET1*-RNAi lines 7 and 9 were crossed with WT *FWA*::*GFP* lines to produce *AP3*::*MET1*-RNAi *FWA*::*GFP* lines 7 and 9 respectively. The *AP3* promoter is expected to drive *MET1*-RNAi expression during petal and anther development therefore these lines

were analysed for reporter expression throughout development of these floral organs. Analysis was performed using fluorescence microscopy.

No GFP was detected in petal primordia or developing petals of the *AP3*::*MET1*-RNAi, *FWA*::*GFP* lines until floral stage 10. However, at floral stage 10 GFP was detected in petals from both lines (**Figure 5.4a and b**). The level of expression was considerably higher than that in petals from the WT *FWA*::*GFP* line (**Figure 5.4c**) but lower than that in the *met1-9 FWA*::*GFP* line (**Figure 5.4d**). At floral stage 12, GFP in petals of *AP3*::*MET1*-RNAi *FWA*::*GFP* lines 7 and 9 was predominant in a trace pattern across the petal (**Figure 5.5f and g**). In contrast, a low level of GFP was present throughout petals from the WT *FWA*::*GFP* line (**Figure 5.4h**), whereas a high level of GFP was present throughout the petal and was also predominant in a trace pattern (**Figure 5.4i**). These findings indicate that hypomethylation is induced in petals of the *AP3*::*MET1*-RNAi lines from Floral stage 10-12.

No GFP was detected in anther primordia or developing anthers of the *AP3*::*MET1*-RNAi *FWA*::*GFP* line 7 and 9 until Floral stage 12 (**Figure 5.4k to o**). At Floral stage 12, GFP was detected in clusters of pollen within a number of anthers from both lines (**Figure 5.4p and q**). In contrast, no GFP was present in pollen from the WT *FWA*::*GFP* line, whereas GFP was detected in pollen throughout all anthers from *met1-9 FWA*::*GFP* line (**Figure 5.4r** and **s**).

GFP was detected in pollen from 33 % and 32 % of anthers from AP3::MET1 RNAi FWA::GFP lines 7 and 9 respectively (n=100). Within these anthers the frequency of pollen expressing *GFP* ranged from a few to a large proportion of the grains (**Figure 5.5a** to **d**). Pollen was further analysed by confocal microscopy. Similarly to the *met1-9 FWA*::*GFP* line, GFP was detected in the vegetative cell and the sperm cells of pollen from both *AP3*::*MET1*-RNAi *FWA*::*GFP* lines, whereas no GFP was detected from pollen from the WT *FWA*::*GFP* line (**Figure 5.5e** to **h**). These findings indicate that hypomethylation is induced in a proportion of the male gametes produced by *AP3*::*MET1*-RNAi lines.

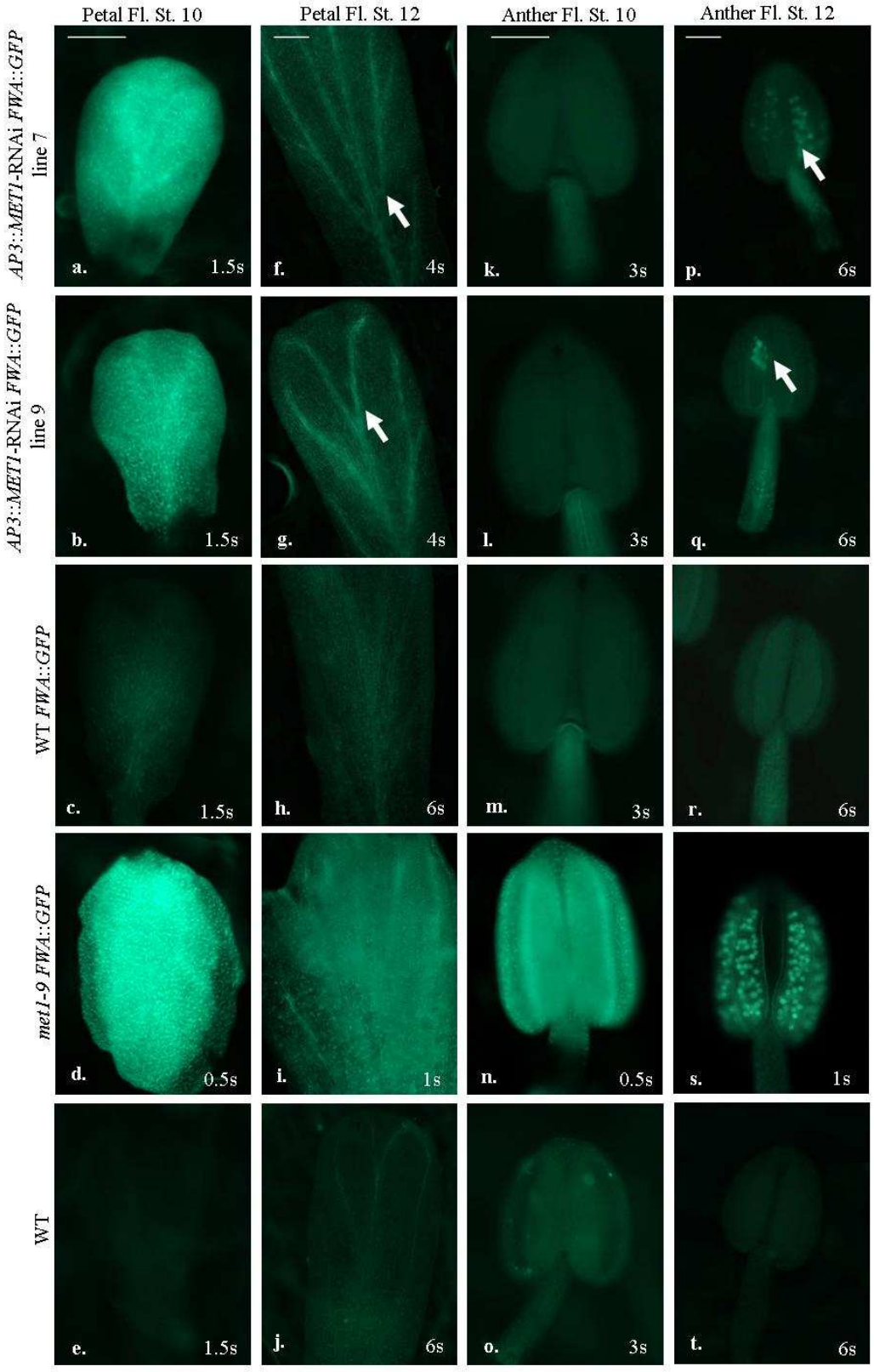


Figure 5.4 *GFP* expression in petals and anthers of the *AP3*::*MET1*-RNAi *FWA*::*GFP* lines. Rows and columns are labelled. In **f.** and **g.** arrows point to the trace pattern of *GFP* expression described above. In **p.** and **q.** arrows point to GFP in pollen within anthers. The number in the bottom right corner of images indicates the exposure time of the photograph in seconds. Scale bar, 100 μm.

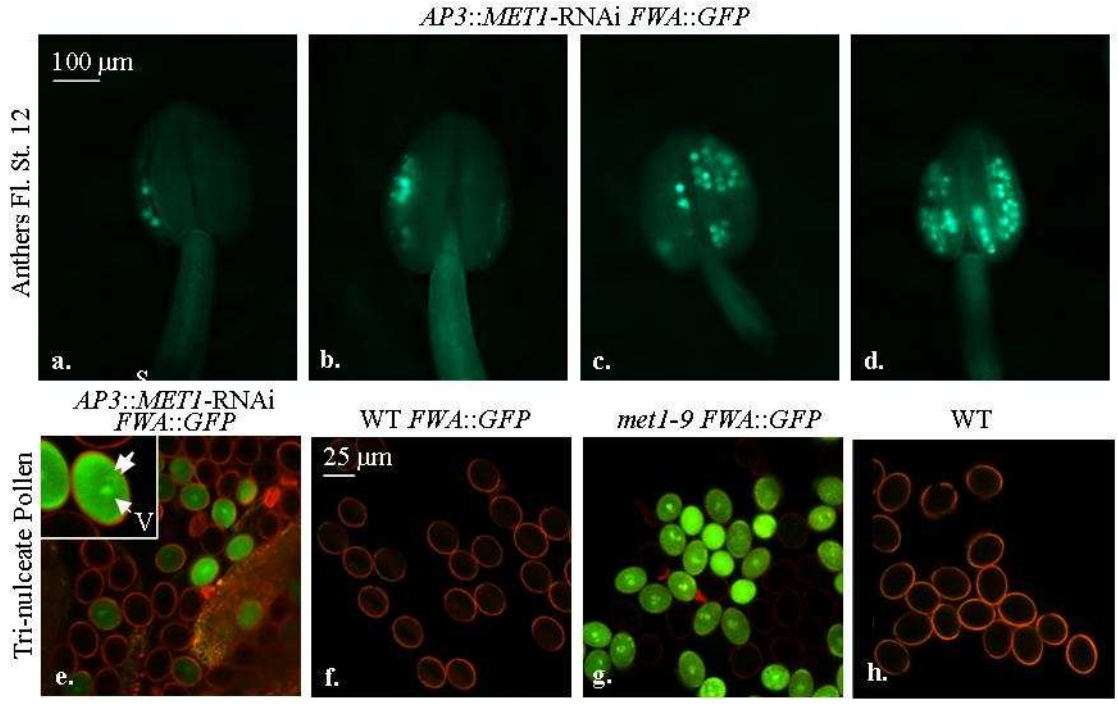


Figure 5.5 *GFP* expression from the anthers *AP3*::*MET1*-RNAi, FWA::GFP lines. **a.** to **d.** show the range in frequency of pollen expressing *GFP* in anthers from *AP3*::*MET1*-RNAi *FWA*::*GFP* lines. **e.** GFP in pollen from *AP3*::*MET1*-RNAi *FWA*::*GFP* lines. Insert shows an enlarged image of a pollen grain. Arrows point to GFP in the vegetative cell (V) and sperm cells (S). **f.**, **g.** and **h.** GFP in pollen from the WT *FWA::GFP* line, the *met1-9 FWA::GFP* line and WT plants respectively.

Inheritance of a hypomethylated genome can cause late flowering due to ectopic *FWA* expression (See Chapter 7). To investigate whether the hypomethylation induced in *AP3*::*MET1*-RNAi lines is transmitted to progeny, WT plants were pollinated by *AP3*::*MET1*-RNAi lines and flowering time of the progeny was analysed. No [WT x *AP3*::*MET1*-RNAi] plants were late flowering and no significant difference was detected between the flowering time of [WT x *AP3*::*MET1*-RNAi] plants and WT plants (H=0.16, d.f.=2, P=0.924). This indicating that the paternally-derived progeny of *AP3*::*MET1*-RNAi lines 7 and 9 were not hypomethylated (**Table 5.3**).

Table 5.3 Flowering time analysis of the progeny from *AP3*::*MET1*-RNAi lines

Lines	Mean Flowering time[a]	S.E.M.	n[b]
[WT x *AP3*::*MET1*-RNAi line 7]	7.9	0.1	55
[WT x *AP3*::*MET1*-RNAi line 9]	7.7	0.2	55
WT	7.7	0.1	46

[a] Flowering-time measured as number of rosette leaves produced before bolting
[b] Number of plants analysed

5.2.3.2 *Methylation analysis of* APG*::*MET1*-RNAi lines*

APG::*MET1*-RNAi lines 13, 15 and 18 were crossed with WT *FWA*::*GFP* lines to produce *APG*::*MET1*-RNAi *FWA*::*GFP* lines 13, 15 and 18 respectively. These lines were analysed for *GFP* expression in mature pollen. No GFP was detected indicating that hypomethylation was not induced in these lines.

5.2.3.3 *Methylation analysis of* SHP2*::*MET1*-RNAi and* At2g20070*::*MET1*-RNAi lines*

SHP2::*MET1*-RNAi *FWA*::*GFP* lines 2 and 8 and *At2g20070*::*MET*-RNAi lines 3, 4, 5 and 11 were crossed with WT to produce *SHP2*::*MET1*-RNAi *FWA*::*GFP* lines 2 and 8 and *At2g20070*::*MET1*-RNAi *FWA*::*GFP* lines 3, 4, 5 and 11 respectively.

SHP2::*MET1*-RNAi *FWA*::*GFP* lines were analysed for *GFP* expression in carpel primordia and throughout carpel development using fluorescence microscopy. The level of GFP in developing carpels of *SHP2*::*MET1*-RNAi *FWA*::*GFP* lines was comparable to that in the WT *FWA*::*GFP* line (**Figure 5.6**). These findings indicate that hypomethylation was not induced during carpel development in *SHP2*::*MET1*-RNAi lines.

SHP2::*MET1*-RNAi *FWA*::*GFP* lines and *At2g20070*::*MET*-RNAi *FWA*::*GFP* lines were also analysed for *GFP* expression in mature ovules using confocal microscopy. The pattern of GFP in the ovules from these lines was identical to that in ovules from the WT *FWA*::*GFP* line; GFP was present only in the central cell. These findings indicate that hypomethylation was not induced in female gametes produced by *SHP2*::*MET1*-RNAi and *At2g20070*::*MET*-RNAi lines..

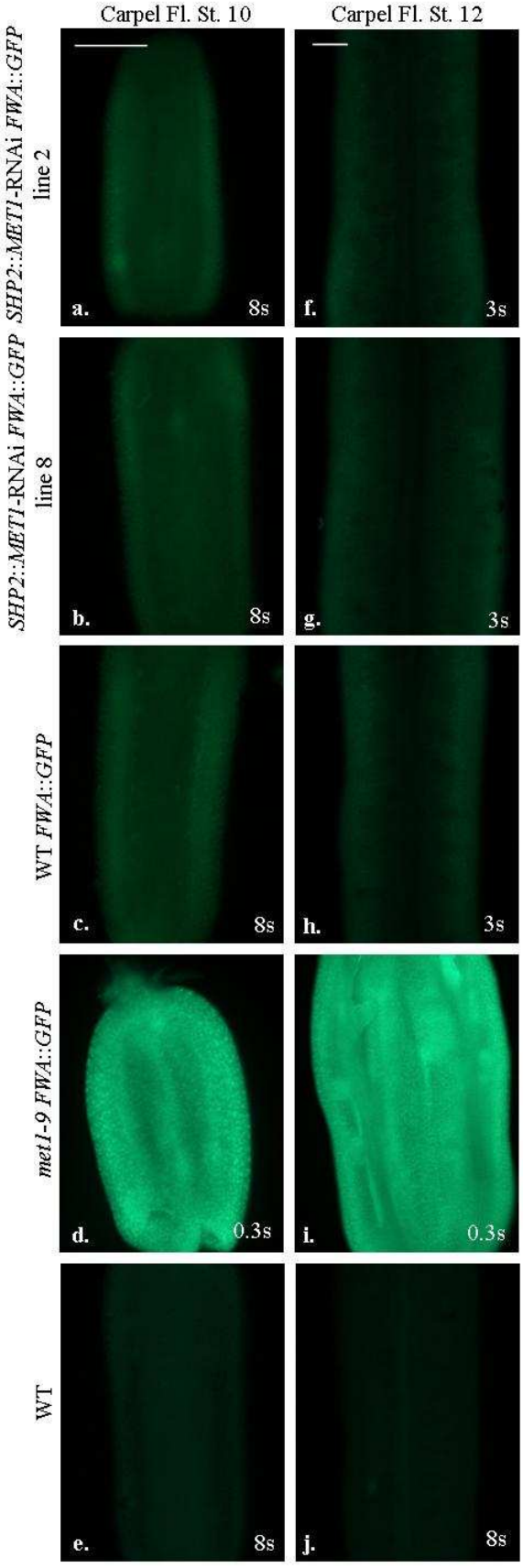


Figure 5.6 *GFP* expression in carpels of the *SHP2*::*MET1*-RNAi *FWA*::*GFP* lines. Rows and columns are labelled. The number in the bottom right corner of images indicates the exposure time of the photographs in seconds. Scale bar, 100 μm.

5.2.4 *Troubleshooting*

Promoter-specific expression of the *MET1*-RNAi transgene was predicted to induce tissue-specific *MET1* suppression and hypomethylation. However, only partial hypomethylation was detected in *AP3*::*MET1*-RNAi lines and no hypomethylation was detected in *APG*::*MET1*-RNAi, *SHP2*::*MET1*-RNAi and *At2g200702*::*MET1*-RNAi lines. Reasons why the predicted tissue-specific hypomethylation was not detected include the possibility that expression of the *MET1*-RNAi transgene does not induce sufficient hypomethylation to invoke ectopic *FWA*::*GFP* expression. Hence, it is possible tissue-specific hypomethylation *is* induced by promoter-specific *MET1*-RNAi expression but the *FWA*::*GFP* reporter is not sufficiently sensitive to detect it.

Alternatively the promoter-specific *MET1*-RNAi transgene *may* not induce the predicted tissue-specific hypomethylation. When constructing the *35S*::*MET1*-RNAi transgene and the promoter-specific *MET1*-RNAi derivatives, sequencing was used to check for errors in all fragments cloned and each cloning step was checked by restriction digestion. However, it is possible that the transgenes were 1) corrupted or fragmented during transformation 2) intact but not expressed or 3) intact and expressed but not strongly enough or for long enough to induce *MET1* suppression and hypomethylation.

5.2.4.1 *Expression of the* FWA*::*GFP *reporter in* 35S*::*MET1*-RNAi lines*

To investigate whether the level of *MET1* suppression induced by *MET1*-RNAi expression is sufficient to invoke ectopic *FWA*::*GFP* expression, *GFP* expression was analysed in lines hemizygous for the *35S*::*MET1*-RNAi transgene and the *FWA*::*GFP* reporter using fluorescence microscopy and confocal microscopy. These lines were produced from crossing the WT *FWA*::*GFP* line with *35S*::*MET1*-RNAi line 1.

GFP was detected in the meristem and in petals, anthers and carpels throughout floral organ development. The level of GFP was comparable to that detected in the *met1-9 FWA*::*GFP* line and thus much higher than that detected in the WT *FWA*::*GFP* line (**Figure 5.6a** to **g**). GFP was also present in pollen from *35S*::*MET1*-RNAi *FWA*::*GFP* plants (**Figure 5.6h**). These findings indicate that the *MET1*-RNAi transgene can induce sufficient hypomethylation to cause detectable ectopic *FWA*::*GFP* expression.

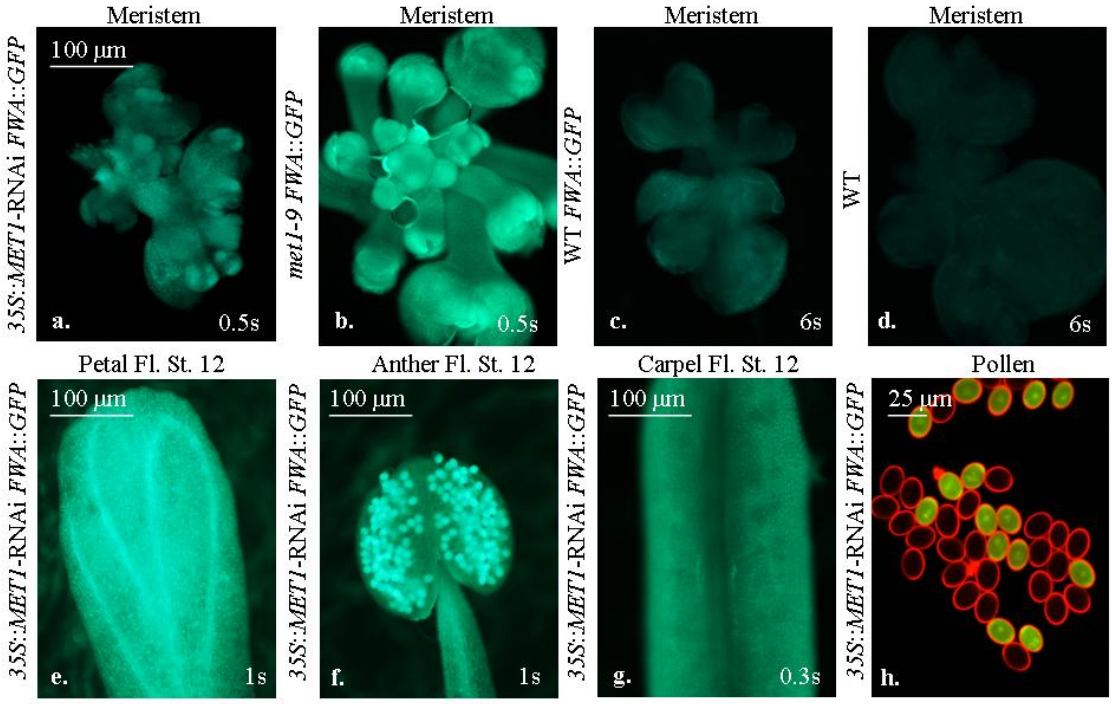


Figure 5.7 *GFP* expression in *35S*::*MET1*-RNAi lines. a., **b** and **c.** GFP in the meristem and young buds of the *35S*::*MET1*-RNAi *FWA*::*GFP* line, the *met1-9 FWA*::*GFP* line and the WT *FWA*::*GFP* line respectively. **d.** Auto-fluorescence from the meristem and young buds of WT plants. **e.**, **f.** and **g.** GFP in petal, anther and carpel from the *35S*::*MET1*-RNAi *FWA*::*GFP* line at Floral stage 12. These images should be compared with those in **Figure 5.4** and **Figure 5.6** which show GFP in petal and anther from the *met1-9 FWA*::*GFP* line and the WT *FWA*::*GFP* line at Floral stage 12. **h.** GFP in pollen from the *35S*::*MET1*-RNAi *FWA*::*GFP* line. This image should be compared with those in **Figure 5.5** showing GFP in pollen from the *met1-9 FWA*::*GFP* line and the WT *FWA*::*GFP* line.

5.2.4.2 *Sequence analysis of the promoter-specific* MET1-*RNAi transgenes*

To test for corruption or fragmentation of the promoter-specific *MET1*-RNAi genes, the transgenes were amplified from promoter-specific *MET1*-RNAi lines and sequenced. Using PCR, two over-lapping fragments spanning the entire length of the transgenes were successfully amplified from *AP3*::*MET1*-RNAi, the *APG*::*MET1*-RNAi, the *SHP2*::*MET1*-RNAi or the *At2g20070*::*MET1*-RNAi lines (**Figure 5.8**). The fragments amplified by PCR2 were sequenced from the 3' and 5' ends. Sequencing revealed no fragment deletions or rearrangement within the transgenes. Additionally, no sequence errors were detected in the 5' region of the tissue-specific promoters, in the junction sequence between the promoters and *MET1*-RNAi fragments, in the sense and antisense *MET1* fragments encoding the *MET1*-RI or in the 3'OCS of the transgenes. These results suggest that the transgene was not corrupted or fragmented during transformation into *Arabidopsis*.

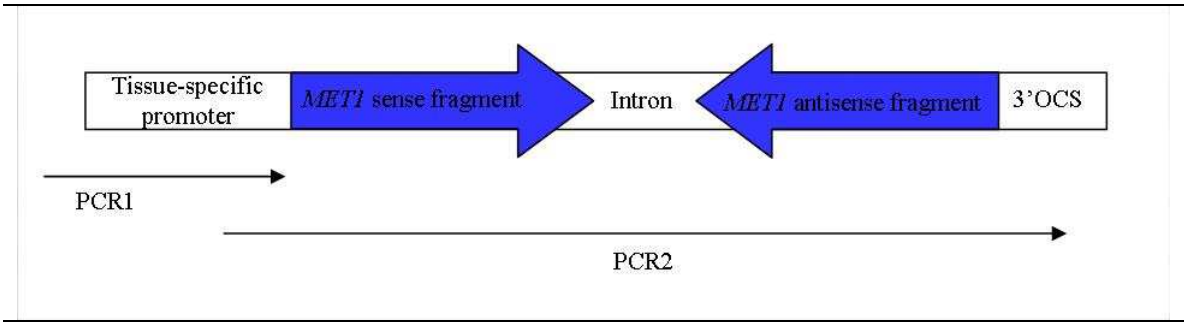


Figure 5.8 Schematic of the promoter-specific *MET1*-RNAi constructs showing the region amplified from transgenic lines by PCR

5.2.4.3 *Expression of the* SHP2*::*MET1-*RNAi transgene*

To investigate whether the *SHP2*::*MET1*-RNAi transgene is expressed, RT PCR was used to test for the presence of *MET1*-RNAi transcript in *SHP2*::*MET1*-RNAi lines. RNA was extracted from carpels at floral stage 10-12 from *SHP2*::*MET1*-RNAi lines 2 and 8. RNA was treated twice with DNAase and concentrated before cDNA synthesis. PCR was then used to amplify the 5' region of the transgene using primers that anneal to the antisense *MET1* fragment and the 3'OCS of the transgene. As the sequence of the transgene and the transgene transcript are indistinguishable, it was necessary to control against the possibility that fragments amplified from the cDNA originated from DNA carried over from the RNA extraction. To this end, the same PCR was performed using different concentrations of the carpel RNA as a template. Any residual DNA in the carpel RNA should be detected by this method. Fragments of the correct size were amplified from carpel cDNA from both *SHP2*::*MET1*-RNAi lines and no fragments were amplified from RNA controls (**Figure 5.8**). These results indicate that RNA extractions were not contaminated with genomic DNA and that *MET1*-RNA transcript was present in carpel tissue from *SHP2*::*MET1*-RNAi lines 2 and 8.

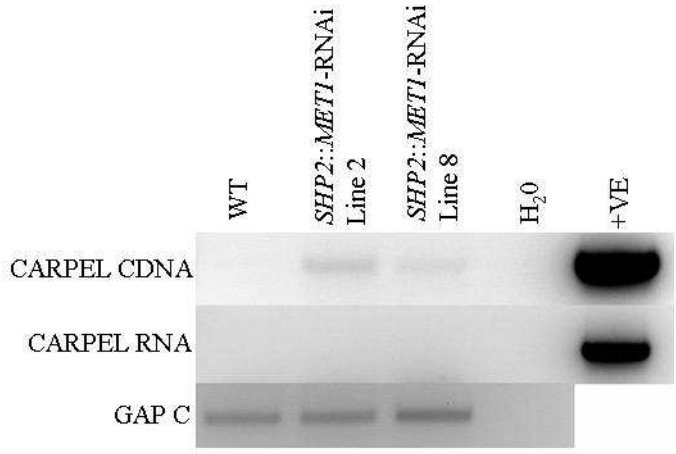


Figure 5.8 RT PCR testing for the presence of *MET1*-RNAi transcript in carpel tissue from *SHP2*::*MET1* RNAi lines. A fragment of the *GAPC* gene was amplified as a control.

5.2.4.4 MET1 *suppression in* SHP2*::*MET1-*RNAi lines*

To investigate whether expression of the *MET1*-RNAi transgene induces *MET1* suppression, semi-quantitative RT PCR was used to analyse the level of *MET1* transcript in carpel tissue of *SHP2*::*MET1*-RNAi line 2 and 8. PCR was performed to amplify a fragment of *MET1* transcript from carpel cDNA synthesised from RNA extracted from carpel tissue at floral stage 10-12. The level of *MET1* transcript in carpel tissue from the transgenic lines was comparable to that from WT plants, indicting that *MET1* is not suppressed at a detectable level in *SHP2*::*MET1*-RNAi lines 2 and 8 (**Figure 5.9**).

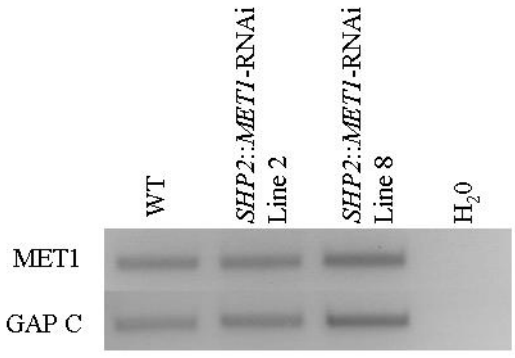


Figure 5.9 Semi-quantitative RT PCR analysing the level of *MET1* transcript in carpel tissue of *SHP2*::*MET1*-RNAi lines. A fragment of the *GAPC* gene was amplified as a control.

5.3 *Discussion*

The aim of the work described in this chapter was to investigate whether a transgenic approach is effective in suppressing *MET1* in a tissue-specific manner to alter genomic imprinting. This section discusses imprinting and methylation in promoter-specific *MET1*-RNAi lines and considers why tissue-specific hypomethylation was not induced as predicted. The experimental approach used to achieve tissue-specific hypomethylation is evaluated and refinements are proposed.

5.3.1 *Imprinting and methylation in promoter-specific* MET1-*RNAi lines*

Imprinting Models 1 and 2 predict that suppression of *MET1* during anther development will release silencing of endosperm-inhibiting genes to be paternally-inherited. Expression of the *MET1*-RNAi gene from the *AP3* promoter was predicted to suppress *MET1* during anther developments and therefore disrupt imprinting in this way. Model 1 also predicts that suppression of *MET1* during the male gametogenesis will cause the same effect on imprinting and expression of *MET1*-RNAi from the *APG* promoter was expected to induce this. Relaxed silencing of endosperm-inhibiting genes reportedly restricts seed size (Adams, S. et al. 2000) and counteracts endosperm-over proliferation of maternal *mea* mutants, thereby rescuing seed abortion in these lines (Spielman, M. et al. 2001). Seed weight and the ability to rescue *mea* abortion were therefore used as proxies for released silencing of endosperm-inhibiting genes derived from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines.

In contrast to predictions, the T3 from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines was equal to or slightly greater than that of WT seed. Additionally, pollen from *AP3*::*MET1*-RNAi and *APG*::*MET1*-RNAi lines did not rescue *mea* seed abortion indicating that silencing is not released from endosperm-inhibiting genes paternally-inherited from these lines.

Models 1 and 2 also predict that *MET1* suppression during carpel development will prevent silencing of maternally-inherited endosperm-promoting genes. Expression of *MET1*-RNAi from the *SHP2* promoter was expected to induce this effect. Additionally, Model 1 predicts that *MET1* suppression during female gametogenesis will have the same outcome and this was expected to be triggered by expressing *MET1*-RNAi from the *At2g20070* promoter. Relaxed silencing of maternally-inherited endosperm promoting genes promotes

endosperm proliferation and increase seed weight (Adams, S. et al. 2000) and therefore seed was also used to assess imprinting in *SHP2*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi lines. Although the average T3 seed from a number of *SHP2*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi lines was slightly greater than that of WT seed, no trend for increased seed weight was apparent. This indicates that silencing is not released from endosperm-promoting genes which are maternally-inherited from these lines.

Reasons why imprinting appears to be unaltered in the promoter-specific *MET1*-RNAi lines analysed include the possibility is that MET1 is not required during anther or carpel development or male and female gametophyte gametogenesis to achieve imprinting. However, this is unlikely as recent findings indicate that *MET1* is required during the gametophyte generation to control imprinting at some loci, including those affecting seed size (Jullien, P. E. et al. 2006b, Xiao, W. et al. 2006a). Alternatively, it is possible that promoter-specific expression of the *MET1*-RNAi construct does not suppress *MET1* and induce tissue-specific hypomethylation as predicted. This is supported by the observation that ectopic expression of the *FWA*::*GFP* reporter is induced in *35S*::*MET1*-RNAi lines but not in *SHP2*::*MET1*-RNAi, *APG*::*MET1*-RNAi or *At2g20070*::*MET1*-RNAi lines. The level of hypomethylation induced by *MET1*-RNAi expression is therefore sufficient to cause ectopic expression of reporter and absence of this in promoter-specific *MET1*-RNAi lines is strong evidence that hypomethylation was either low or not induced in these lines. Consequently, no valid conclusions relating to the effect on imprinting caused by *MET1* suppression can be made from the analysis of these lines.

However, ectopic *FWA*::*GFP* expression was detected in petals and approximately one sixth of pollen from *AP3*::*MET1*-RNAi lines indicating that tissue-specific *MET1* suppression and hypomethylation is induced, albeit not in as broad a developmental window as predicted. The finding that pollen from *AP3*::*MET1*-RNAi lines is hypomethylated but that imprinting of genes paternally-inherited from these lines was not altered, could indicate that *MET1* is not required in the male gametophyte generation to achieve imprinting. However, as suggested above, this unlikely.

Alternatively, it is possible that the hypomethylation detected in the pollen was not maintained or transmitted to seed paternally-derived from *AP3*::*MET1*-RNAi lines. This is supported by the observation that [WT x *AP3*::*MET1*-RNAi] plants were not late-

flowering. *FWA* is ectopically expressed during embryogenesis and vegetative development of *met1* heterozygotes which are paternally-derived from hypomethylated *met1* mutants and these *met1* heterozygotes display an *FWA*-dependent late-flowering phenotype (See Chapter 7 & Kinoshita, T. et al. 2004). Additionally, *FWA* is ectopically expressed in seed paternally-derived from *met1-3* heterozygotes, which exhibit reduced *MET1* activity only during the gametophyte generation (Jullien, P. E. et al. 2006b). This suggests that hypomethylation in the male gametophyte generation can be paternally-transmitted and induce an *FWA*-dependent late-flowering phenotype. The absence of this in [WT x *AP3*::*MET1*-RNAi] plants indicates that hypomethylated genomes were not paternally-transmitted from *AP3*::*MET1*-RNAi lines.

Additional evidence that hypomethylation was not paternally-transmitted from *AP3*::*MET1*-RNAi lines is that the *FWA*::*GFP* reporter was not ectopically expressed in seed paternally-derived from these lines. Jullien et al (2006) reported that the *FWA*::*GFP* reporter is expressed in pollen from *met1-3* heterozygous plants and in seed paternally-derived from the plants. This indicates that ectopic *FWA*::*GFP* expression induced by hypomethylation during the male gametophyte generation can be maintained after fertilization. The absence of this in seed from *AP3*::*MET1*-RNAi lines again suggests that hypomethylated genomes were not paternally-transmitted from these lines. As a consequence, no valid conclusions relating to the effect on imprinting caused by *MET1* suppression can be made for the analysis of these lines.

One explanation why hypomethylated genomes were not paternally-transmitted from *AP3*::*MET1*-RNAi lines is that hypomethylated pollen was out-competed by its normally-methylated siblings. In support of this, the proportion of *met1-9* homozygotes derived from self-fertilised *met1-9* heterozygotes was frequently lower than expected; appropriately 1:16 as opposed to 1:4. This suggests a problem with the transmission of hypomethylated *met1-9* gametes. Additionally, the paternal inheritance of hypomethylated *met1* genomes increases seed abortion (Xiao, W. et al. 2006b). Any paternally-derived progeny of *AP3*::*MET1*-RNAi lines which inherited hypomethylated genomes may therefore have aborted or failed to germinate.

Additionally, it should also be noted that only a small number of lines were selected from analysis base on their T2 seed weight. Effects of hypomethylation reportedly become

increasingly severe following successive generations of inbreeding (Finnegan, E. J. et al. 1996, Kakutani, T. et al. 1996). It is therefore possible that any effects of hypomethylation in promoter-specific *MET1*-RNAi lines will only be evidence in later generation. Finally, it should also be noted that the promoter-specific *MET1*-RNAi transgene copy number was not assessed in this analysis. Lines carrying a single copy of an inverted repeat transgene reportedly silence endogenous gene more effectively than multi-copy insertion lines (Kerschen, A. et al. 2004). Transgene copy number may therefore have influenced the level of hypomethylation and altered imprinting in these test lines.

5.3.2 *Hypotheses suggesting why promoter-specific* MET1-*RNAi transgenes did not induce tissue-specific hypomethylation as predicted*

Expression of *MET1*-RNAi from tissue-specific promoters was predicted to induce *MET1* suppression and hypomethylation in a developmental window mirroring that in which the promoter can drive expression. Detection of hypomethylation in pollen from *AP3*::*MET1*-RNAi lines indicates that promoter-specific expression of the *MET1*-RNAi construct can induce *MET1* suppression and hypomethylation. However, not in the profile predicted. There are several possible reasons why this could be.

Firstly, expression of the *MET1*-RNAi gene might require several generations of cell division to induce a level of hypomethylation sufficient to alter gene expression. Consistent with this, the *AP3* promoter drives expression from floral stage 3-10 (Jack, T. et al. 1992), yet hypomethylation was not apparent until floral stage 10, i.e. at the latter end of this developmental window. *met1-2* mutants have a 50 % reduction in DNA methylation but do not display any phenotypic abnormalities, indicating that a reduction in methylation can be tolerated without significantly altering the expression of developmental genes (Kankel, W. et al. 2003). It is also possible that expression of *MET1*-RNAi from the *AP3* promoter induced *MET1* suppression at floral stage 3, but the resultant level of hypomethylation was insufficient to alter gene expression until floral stage 10. The *SHP2*, *APG* and *At2g20070* promoters drive expression in a narrower developmental window than *AP3*, it is therefore possible hypomethylation was not detected in *SHP2*::*MET1*-RNAi, *APG*::*MET1*-RNAi and *At2g20070*::*MET1*-RNAi lines because the window of *MET1*-RNAi was insufficiently wide to induce hypomethylation at a detectable level.

Secondly, the tissue-specific promoters may be too weak to induce *MET1* suppression. In agreement with this, *MET1* transcript levels were not reduced in *SHP2*::*MET1*-RNAi lines yet transgene expression was detected. It is recognised that RNAi constructs driven by weaker promoters are less effective than those driven by strong promoters (Chuang, C. F. and Meyerowitz E.M. 2000). This is exemplified by an attempt to suppress the transcription factor *TjTisII* in a root-knot nematode feeding from roots of transgenic plants which express a *TjTisII*-RI transgene (Fairbairn, D. J. et al. 2007). TjTis11 suppression was induced in nematodes fed on roots from 35S::*TjTis11*-RNAi plants but not in those fed on roots from plants carrying the *TjTis11*-IR expressed from a root-specific *TobRB7* promoter. The authors suggest that this is due to the weakness of this promoter and concluded that a hairpin-containing transgene is not sufficient to induce silencing unless it is linked to a strong promoter. *met1* heterozygotes can have a phenotype similar to WT plants (Saze, H. et al. 2003), indicating that a 50 % reduction in *MET1* expression can be tolerated without significantly altering the expression of developmental genes. It is therefore possible that *AP3*, *SHP2*, *APG* and *At2g20070* promoters are not powerful enough to express *MET1*-RNAi at a level sufficient to induce hypomethylation.

Thirdly it is possible that the tissue-specific promoters do not drive expression in the appropriate tissues. Although the developmental windows in which the promoters are capable of driving expression is well reported in the literature (See section **5.2.1.1**), it is advisable to track the expression of independent transgenes as they may be have a unique expression profiles. Additionally, it is possible that the promoters do not drive expression at the appropriate stage of the cell cycle, i.e. when *MET1* is expressed. The approach described here lacked a transgene expression tracking system therefore the tissue-specific and cycle-cell specific expression profile of the transgenes could not be assessed.

5.3.3 *Using promoter-specific RNAi-induced* MET1 *suppression to investigate and manipulate genomic imprinting*

It was proposed that an approach enabling tissue-specific *MET1* suppression and hypomethylation could be used to validate the proposed models of genomic imprinting and to manipulate seed size (Chapter 1, sections **1.4.2.6.** and **1.5.**). The detection of hypomethylation in pollen from *AP3*::*MET1*-RNAi lines indicates that tissue-specific *MET1*-suppression and hypomethylation can be induced by expression a *MET1* inverted-

repeat fragment from tissue-specific promoters. This approach now requires improvement to achieve targeted sex-specific hypomethylation.

The analysis described here suggests that promoter choice is critical for achieving tissue-specific *MET1*-suppression and hypomethylation. Promoters expressing the *MET1*-RNAi transgene may not induce hypomethylation in the developmental window in which they drive expression. Additionally, *MET1* suppression and hypomethylation may only be induced where the *MET1*-RNAi transgene is expressed from a strong promoter. Further work should focus on identifying strong promoters which drive expression prior to and during the stage when hypomethylation is required. Novel promoter-specific *MET1*-RNAi transgenes should also include a reporter to enable transgene expression to be easily tracked. Subsequently, novel promoter-specific *MET1*-RNAi lines should be analysed for hypomethylation and altered imprinting using the approaches described above and transgene expression should also be tracked. If appropriate promoters cannot be identified, it may be possible to achieve the same effect using multiple constructs, each driving *MET1*-IRs from different promoters.

Despite considerable effort to determine how efficiency of transgene-induced RNAi can be improved, many studies still report a high level of variation in the effectiveness of this approach (McGinnis, K. et al. 2007, Wang, T. et al. 2005). This underlines the need for large-scale screens to identify effective lines. In this study, only a small number of lines were screened for hypomethylation, which may in part explain why hypomethylation was only identified in a small number of lines. Future trials should screen a larger number of lines for hypomethylation. The *FWA*::*GFP* reporter is a useful tool to signal tissue-specific hypomethylation but must be crossed to transgenic lines before screening can begin. This process is time consuming. To address this problem, future constructs should be engineered to carry both the promoter-specific *MET1*-RNAi gene and a reporter. **Figure 5.10** show the plasmid map of an example construct. Alternatively promoter-specific *MET1*-RNAi transgenes could be transformed directly into *FWA*::*GFP* reporter lines.

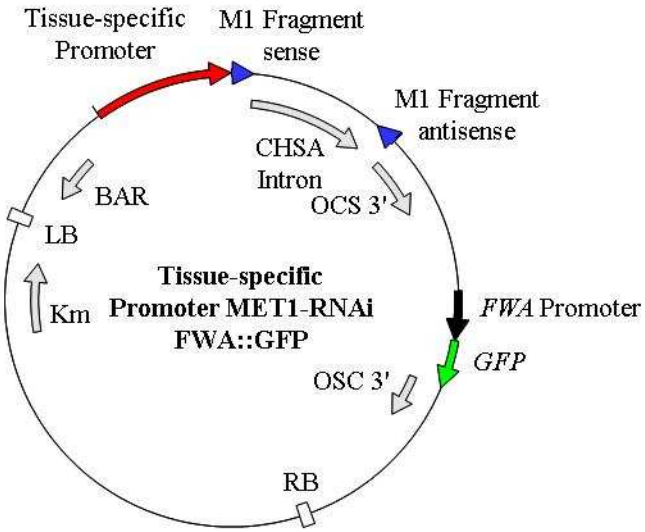


Figure 5.10 Plasmid map of the proposed promoter-specific *MET1*-RNAi *FWA*::*GFP* construct

Future trials should also consider whether successive generations of inbreeding and transgene copy number effect the level of hypomethylation and altered imprinting achieved in transgenic lines.

Chapter 6 - *The effect of hypomethylation on seed development in tobacco*

6.1 *Introduction*

The ability to manipulate seed size has significant agricultural implications, for example it provides the potential to increase yield and to develop varieties/cultivars that can be grown in stress-prone environments (Chapter 1, section **1.5**). The small seed phenotype of [WT x *MET1*as] seed and the large seed phenotype of [*MET1*as x WT] seed, indicates that DNA methylation plays an important role in controlling the parent-of-origin-specific expression of genes affecting seed size in *Arabidopsis*. In maize, the parent-of-origin-specific expression of *FIE1* and *FIE2* is correlated with the parent-of-origin-specific methylation of DRMs associated with these genes (Gutierrez-Marcos, J. F. et al. 2006). This suggests that DNA methylation could play an important role in controlling imprinted gene expression in a wide range of plant species. This raises the exciting prospect that seed size can be manipulated in agriculturally-important crop species via the parent-of-origin-specific inheritance of hypomethylated genomes. However, the effect on seed size from this approach has only been demonstrated in *Arabidopsis*. The aim of the work described in this chapter was to investigate the effect on seed size from the parent-of-origin-specific inheritance of hypomethylated genomes in a second species, namely tobacco (*Nicotiana tabacum*).

Tobacco was chosen as a test species for several reasons. Firstly, although tobacco is dicotyledonous, tobacco seed has characteristics resembling seed of the more agriculturally-significant monocotyledonous plants. For example, in contrast to the transient endosperm of *Arabidopsis* which is almost entirely consumed during seed development, multiple layers of endosperm are present in mature seed from tobacco (Avery, G. S. 1933). Therefore, the endosperm of tobacco has greater resemblance to the persistent endosperm of cereals than does *Arabidopsis* (Chapter 1, section **1.2.2**). Consequently, investigating the effects of hypomethylation on seed development in tobacco could help predict the effects of hypomethylation on seed development in cereals.

Secondly, due to the limited number or oil seed crops available and the global push to switch to more sustainable resources, tobacco seed could become an increasingly valuable resource because it has both a high oil content and useful oil composition (Giannelos, P. N. et al. 2002). For example, it has already been demonstrated that tobacco seed oil methyl

ester can be used as a substitute for diesel fuel (Usta, N. 2005). The ability to manipulate seed size in tobacco could therefore prove valuable, since its current very small size is agronomically undesirable.

Thirdly, tobacco was chosen as a test species because of the availability of hypomethylated *35S*::*NtMET1*as tobacco lines which carry a transgene consisting of an antisense fragment of *NtMET1* cDNA 5' fused to the CaMV *35S* promoter (Nakano, Y. et al. 2000). *35S*::*NtMET1*as lines are hypomethylated and have phenotype abnormalities which include reduced leaf size, short internodes and floral distortions (Nakano, Y. et al. 2000). However, no seed phenotype has been reported for these lines.

If seed size can be manipulated in agriculturally important crop species by the parent-of-origin-specific inheritance of hypomethylated genomes, it would be advantageous to develop a transgenic approach that can be used to suppress MET1 activity in a variety of plant species. In Chapter 1 (section **1.5**), it was hypothesised that this could be achieved using a transgene that recognises the conserved DNA methyltransferase motif sequences and triggers the suppression of any *MET1*-type DNA methyltransferase. In order to test this hypothesis, a further aim of the work described in this chapter was to investigate whether or not transgenes designed to suppress *MET1* in *Arabidopsis* (*AtMET1*) can trigger *MET1* suppression in tobacco (*NtMET1*).

6.2 *Results*

6.2.1 *Effects of hypomethylation on seed size in tobacco*

The effect of hypomethylation on seed size was investigated by analysing the weight of seed produced from *35S*::*MET1*as lines.

6.2.1.1 *F2 seed weight of* 35S*::*NtMET1*as lines*

Selfed seed from seven *35S*::*NtMET1*as lines was compared with a control line which had been transformed with an empty pBI121 vector. The average individual seed weight from the majority of *35S*::*NtMET1*as lines was reduced compared to the control lines (**Figure 6.1**). Notably, a low seed weight was detected in lines which did not display an abnormal vegetative/floral phenotype or have a detectable reduction in DNA methylation, as well as lines which were phenotypically abnormal and hypomethylated (Figure 6.1. & Nakano, Y. et al. 2000).

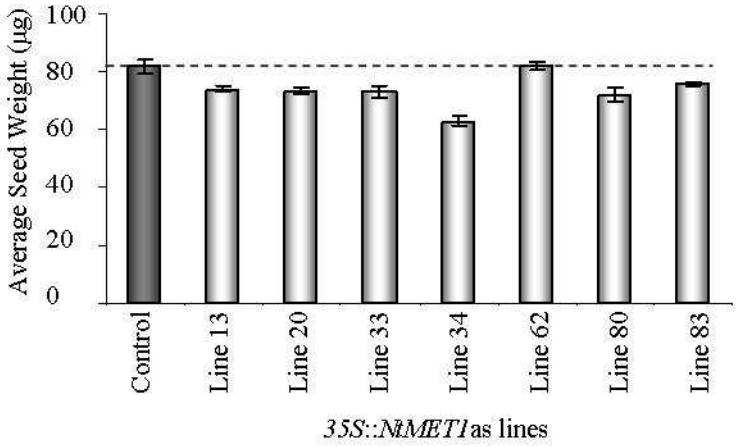


Figure 6.1 Average weights of F2 seed from *35S::NtMET1*as lines. Average seed weight calculated as the mean of the average weight of 50 seed from 5 batches of per line. Error bars show the standard error.

6.2.1.2 *Seed pod size and seed weight of progeny from reciprocal crosses between* 35S*::*NtMET1*as and WT plants*

To test whether or not the parent-of-origin-specific inheritance of hypomethylated genomes affects seed development in tobacco, plants of the *35S*::*NtMET1*as line 62 were crossed reciprocally with WT plants. The total weight of seed per pod produced from these crosses was compared with that produced from self-fertilized WT and *35S*::*NtMET1*as line 62 plants. The *35S*::*NtMET1*as line 62 was used for this analysis because it reportedly carries a single copy of the transgene, displays phenotype characteristics of hypomethylation and has the greatest reduction in methylation of all the lines available (Nakano, Y. et al. 2000). In accordance with this, *35S*::*NtMET1*as line 62 plants grown for this analysis displayed phenotype abnormalities; these include a reduced leaf size and internode length, the absence of petioles and the direct fusion of leaves to the stem, bleached and malformed flowers, an increased number of anthers and multiple fused carpels (**Figure 6.2**). These phenotypes are similar to those described for other hypomethylated *35S*::*NtMET1*as lines (Nakano, Y. et al. 2000).

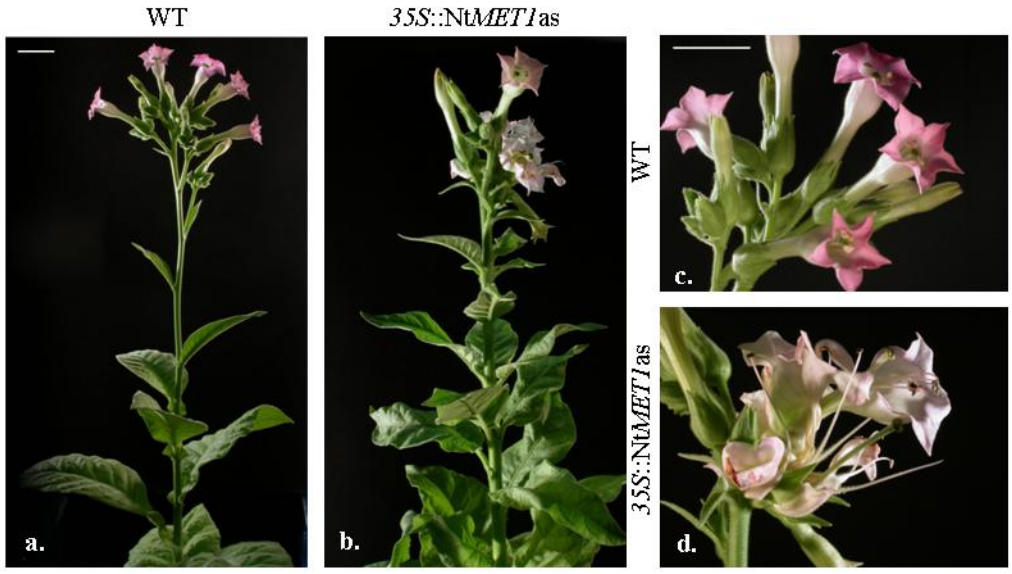


Figure 6.2 The vegetative and floral phenotype of WT and *35S*::*NtMET1*as line 62 plants. a. and **c.** A WT plant and **b.** and **d.** A *35S*::*NtMET1*as plant. Scale bar, 3 cm.

Five F3 *35S*::*NtMET1*as plants were reciprocally crossed with five WT plants to produce [WT X *35S*::*NtMET1*as] and [*35S*::*NtMET1*as X WT] seed. Five flowers were pollinated per plant and all other flowers and buds were removed. To produce *35S*::*NtMET1*as (+) and WT (+) seed, five different F3 *35S*::*NtMET1*as and WT plants were emasculated and then manually self-pollinated. This method of self-fertilization was used to control against any differences in seed set which may occur when the plants are naturally or manually pollinated. Again, five flowers were pollinated per plant and all other flowers and buds were removed. Mature pods from each cross were harvested and the total seed per pod was weighed.

[WT X *35S*::*NtMET1*as] seeds pods were plump and well developed and closely resembled WT (+) seed pods. In contrast, [*35S*::*NtMET1*as X WT] seed pods were small and shrivelled and closely resembled *35S*::*NtMET1*as (+) seed pods (**Figure 6.3a**). On average, the total weight of seed from [WT X *35S*::*NtMET1*as] seed pods was 23 % less than that from WT (+) seed pods, whereas, the total weight of seed from [*35S*::*NtMET1*as X WT] seed pods was 88 % less than that from WT (+) seed pods and approximately equal to that from *35S*::*NtMET1*as (+) seed pods (**Figure 6.3b**).

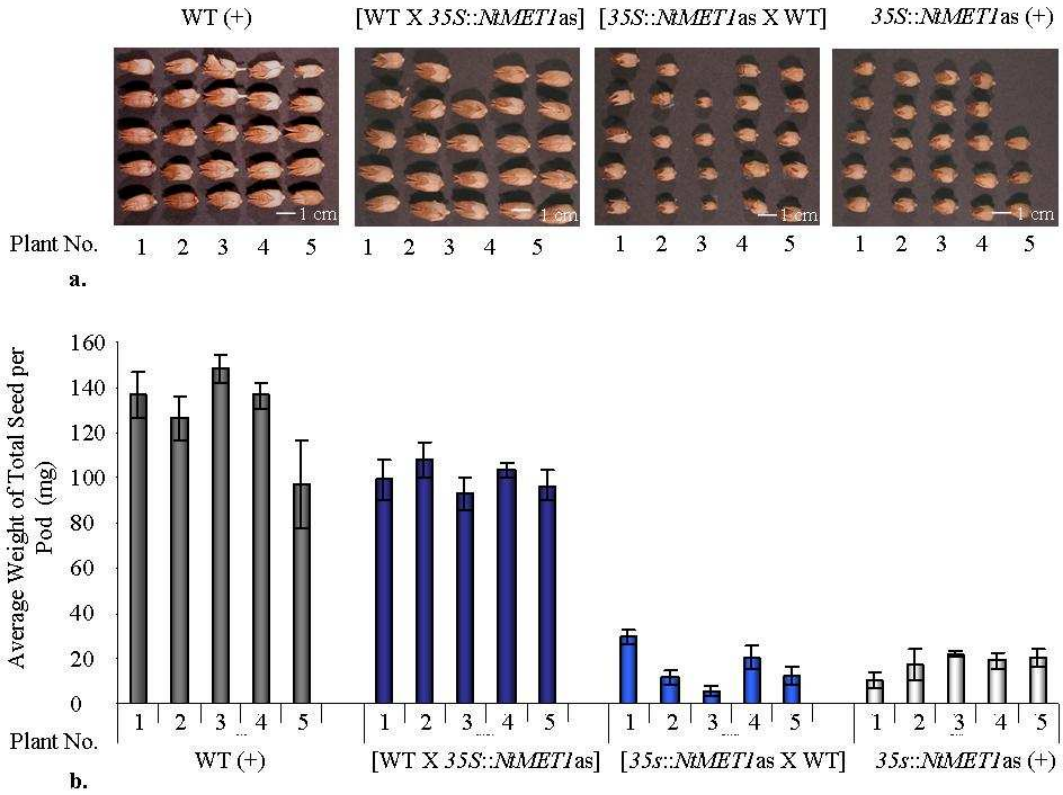


Figure 6.3 The seed pod size and total weight of seed per pod produced from reciprocal crosses between WT and *35S::NtMET1*as plants. a. Pods sizes collected from each cross. In each image, the pods collected from each plant are organised in rows. Where pods are absent the cross failed. **b.** The mean total weight of seed per pod for each plant per cross. The error bar shows standard error. The five plants per cross correspond to the same five plants per cross shown in **a.**

It was expected that seed from the *35S*::*NtMET1*as X WT cross would weigh more than that from the WT X *35S*::*NtMET1*as and WT (+) crosses and that seed from the WT X *35S*::*NtMET1*as cross would be less than that from the *35S*::*NtMET1*as X WT and WT (+) crosses. The weight of seed from the *35S*::*NtMET1*as X WT cross was therefore much less than predicted. It was envisaged that this could either by caused by a reduction in the average weight of individual seed or a reduction in the number of seed set from this cross. Moreover, it was observed that seed from a single pod was not uniform in size and that the proportion of different sized seeds per pod varied in each cross. To investigate this further, seed from each pod was passed through two sieves with different mesh sizes to separate the seed into three size groups. Seed collected on a 0.42mm mesh is referred to as 'large' seed, seed collected on a 0.25mm mesh is referred to 'small' seed and seed that passed through a 0.25mm mesh is referred to as 'shrivelled' seed (**Figure 6.9a**). The number of 'large', 'small' and 'shrivelled' seed and the average weight of 'large' and 'small' seed

from each pod were determined. Subsequently, the average number of 'large', 'small' and 'shrivelled' seed and the average weight of 'large' and 'small' seed from each cross were calculated (**Figure 6.9b and c)**.

35S::*NtMET1*as (+) and *35S*::*NtMET1*as X WT crosses produced more than 50 % fewer seeds per pod than WT (+) and WT X *35S*::*NtMET1*as crosses. Moreover, the majority of seed from these crosses was 'shrivelled' (59 % and 69 % respectively) and only a small proportion of the seed was 'large' (21 % and 13 % respectively). In contrast, the majority of seed from WT (+) and WT X *35S*::*NtMET1*as crosses was 'large' (82 % and 74% respectively) and only a small proportion was 'shrivelled' (9 % and 15 % respectively) (**Figure 6.9b**). However, as expected, the average weight of 'large' and 'small' seed from the *35S*::*NtMET1*as X WT cross was greater than that from WT X *35S*::*NtMET1*as WT (+) crosses and the average weight of 'large' and 'small' seed from the WT X *35S*::*NtMET1*as cross was less than that from *35S*::*NtMET1*as X WT and WT (+) crosses. Additionally, the average weight of 'large' and 'small' seed from the *35S*::*NtMET1*as (+) cross was slightly less that than from the WT (+) cross (**Figure 6.9c**).

'Small' and 'shrivelled' seed from all crosses failed to germinate on plant media suggesting that the seed had aborted. To investigate the cause of seed abortion, small and shrivelled seed was dissected and the content examined (data not shown). The majority of seed either contained a fleshy but under-developed embryo surrounded by endosperm or appeared empty. Occasionally, the shrivelled remains of an embryo could be seen inside the empty seed cavity.

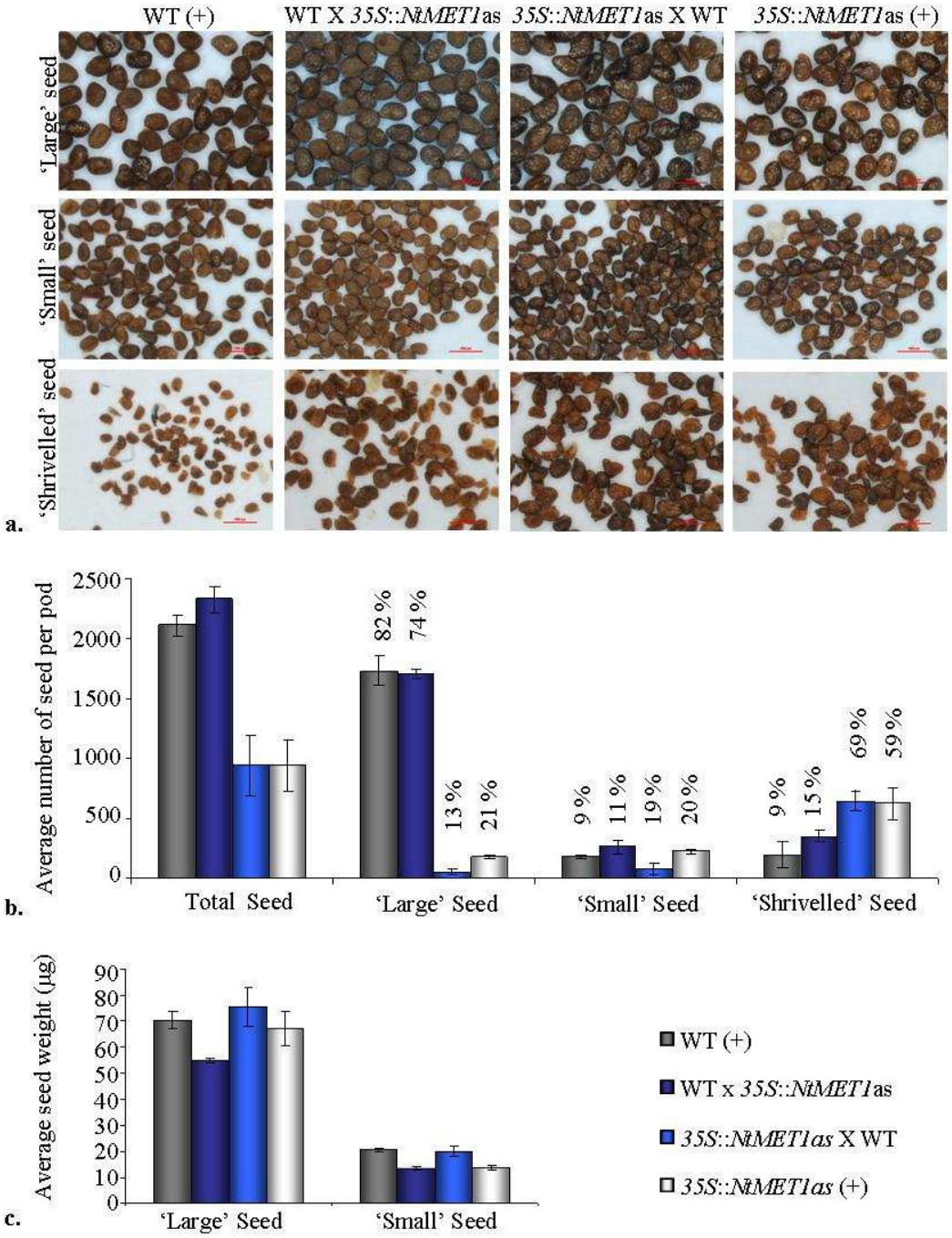


Figure 6.4 Analysis of seed from crosses between WT and *35S*::*NtMET1*as plants. a. images of 'large', 'small and 'shrivelled' seed from each cross. **b.** the average total number of seed produced from each cross and the average number of 'large', 'small and 'shrivelled' seed produced from each cross. Percentage figures give the average of seed from each size range from each cross (as stated in text). **c.** the average weight of 'large' and 'small' seed from each cross. All averages are calculated as the mean of the average number/weight of five pods per plants from five plants per cross.

6.2.2 *Production and analysis of tobacco* 35S*::*AtMET1*as lines*

To test whether *NtMET1* suppression and hypomethylation can be induced by the expression of antisense-orientated fragments of *AtMET1*, transgenic tobacco lines carrying *35S*::*AtMET1*as constructs were produced and analysed for hypomethylation.

6.2.2.1 *Construction of* 35S*::*AtMET1*as constructs*

Three *35S*::*AtMET1*as constructs (*35S*::*AtMET1*.F1as, *35S*::*AtMET1*.F2as, *35S*::*AtMET1*.F3as) were made and transformed in to *A. tumefaciens* by Dr. S. Tiwari (University of Bath). Each construct includes an antisense-orientated *AtMET1* cDNA fragment 5' to the CaMV *35S* promoter. The antisense-orientated *AtMET1* cDNA fragments included in the *35S*::*AtMET1*.F1as and *35S*::*AtMET1*.F2as constructs span the entire catalytic domains of *AtMET1*, as well as a large proportion of the amino terminal domain. In contrast, the *AtMET1* cDNA fragments included in the *35S*::*AtMET1*.F1as spans a region of the *AtMET1* catalytic domain which includes only the catalytic motifs II, IV, VI, VII, VIII and IX (**Figure 6.5**).

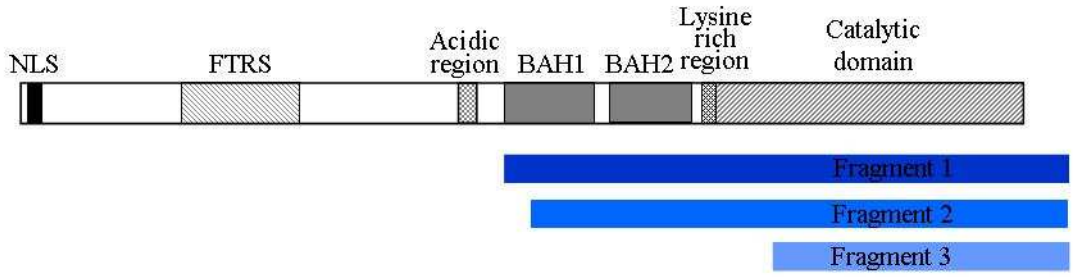


Figure 6.5 Schematic of *AtMET1* functional domains and the antisense-orientated *AtMET1* cDNA fragments included in the *35S*::*AtMET1*as constructs. *35S*::*AtMET1*.F1as includes fragment 1, *35S*::*AtMET1*.F2as includes fragment 2 and *35S*::*AtMET1*.F3as includes fragment 3.

6.2.2.2 *Transformation of the 35S::AtMET1as transgenes into tobacco*

35S::*AtMET1*.F1as, *35S*::*AtMET1*.F2as and *35S*::*AtMET1*.F3as constructs transformed into *A. tumefaciens* and subsequently the transgenes were transformed into tobacco by leaf disk transformation. Transgenic plantlets were selected on BASTA containing growth media. 6 *35S*::*AtMET1*.F1as plantlets, 9 *35S*::*AtMET1*.F2as plantlets and 9 *35S*::*AtMET1*.F3as plantlets were recovered and transferred to soil. All plantlets were successfully taken to maturity. Mature T1 *35S*::*AtMET1*.F1as, *35S*::*AtMET1*.F2as and *35S*::*AtMET1*.F3 lines were all phenotypically similar to WT plants.

6.2.2.3 *DNA methylation in* 35S*::*AtMET1*as lines*

Southern hybridisation was performed to analyse the methylation status of a selection of *35S*::*AtMET1*as lines. DNA was isolated from leaf tissue, digested with either *Hpa*II or *Msp*I and then was probed with a fragment of the *Tto1* retrotransposon, which is present in multiple forms in the tobacco genome (Hirochika, H. 1993). *Hpa*II and *Msp*I both cleave at CCGG sites but *Hap*II cleavage is inhibited by cytosine methylation (McClelland, M. et al. 1994). Thus, cleavage with *Msp*I and the inhibition of cleavage with *Hpa*II indicates that CCGG sites are methylated.

WT DNA is largely digested by *Msp*I but following digestion with *Hap*II an abundance of large fragments remain undigested. This indicates that a considerable proportion of CCGG sites are methylated in WT DNA. In contrast, DNA from the *35S*::*NtMET1*as line 62 is equally digested by *Hap*II and *Msp*I which supports the claim by Nakano, Y. et al. (2000), that this line is hypomethylated. Similarly, *35S*::*AtMET1*.F1as lines 1 and 2 and *35S*::*AtMET1*.F1as line 2 are equally digested by *Hap*II and *Ms*pI indicating that these lines are hypomethylated. In contrast, the digestion pattern of DNA from *35S*::*AtMET1*.F3as line 1 is similar to that of WT DNA, suggesting that the methylation level in this line is similar to that of WT plants.

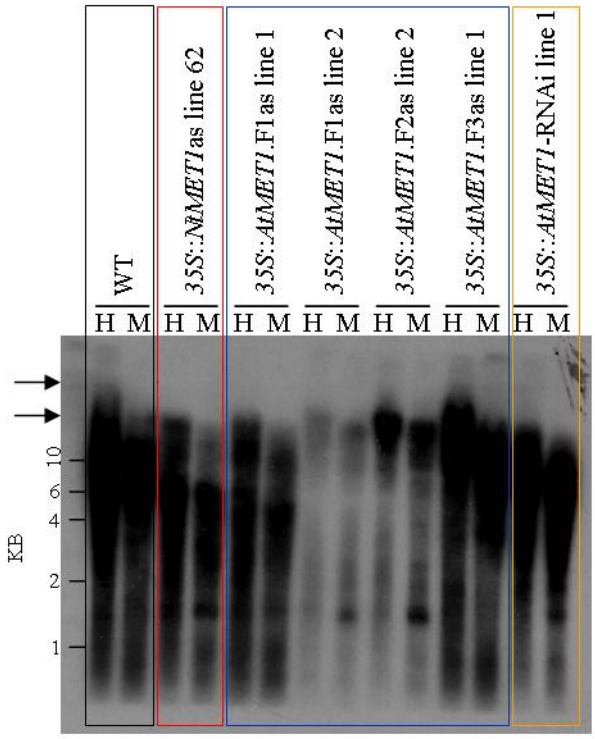


Figure 6.6 Methylation analysis of *35S::AtMET1*as lines using Southern hybridisation. 4.5 µg of DNA per lane was digested with *Hap*II (H) or *Msp*I (M), transferred to a nylon membrane after denaturation and subject to hybridisation by probing with a *Ttol* cDNA fragment (nucleotide positions 263-565).

6.2.3 *Design, construction and analysis of* 35S*::*NtMET1*-RNAi and* 35S*::*AtMET1*-RNAi lines*

To test whether an inverted repeat of an *AtMET1* cDNA fragment can trigger RNAi-induced suppression of *NtMET1*, transgenic tobacco lines carrying a *35S*::*AtMET1*-RNAi gene were produced and analysed. Additionally, and in order to test the effect of RNAi-induced suppression of *NtMET1*, lines carrying a *35S*::*NtMET1*-RNAi gene were produced and analysed.

6.2.3.1 *Choice of* NtMET1 *and* AtMET1 *IR sequences*

An alignment of *NtMET1* and *AtMET1* cDNA sequences identified a region of high sequence homology within the methyltransferase catalytic domain to use as an IR sequence to trigger RNAi-induced *NtMET1* suppression. The selected fragment is 352 bp in length and has 75 % nucleotide sequence homology between *AtMET1* and *NtMET1*. Additionally, this fragment includes the conserved DNA methyltransferase motifs I, II and part of IV, including codons encoding the prolyl-cysteinyl doublet which is predicted to form the catalytic site (Finnegan, E. J. and Dennis, E. S. 1993) (**Figure 6.7**).

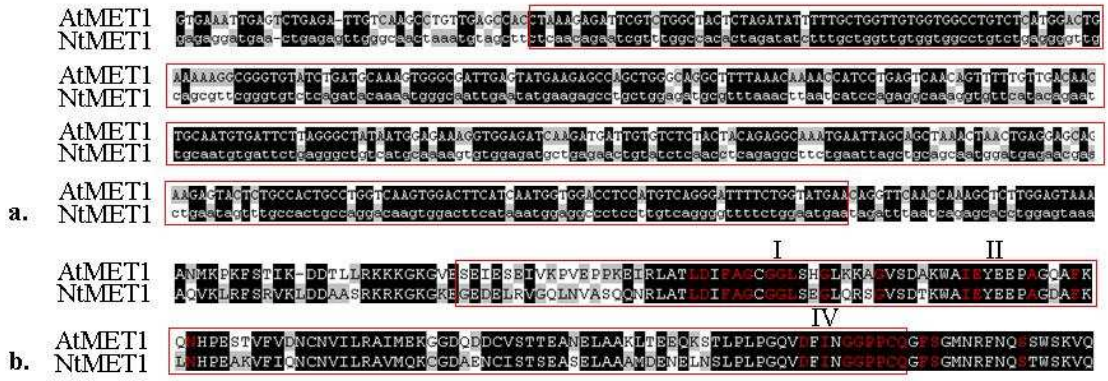


Figure 6.7 The *AtMET1* and *NtMET1* fragments selected as an IR to trigger RNAi-induced suppression of *NtMET1*. a. a region of an alignment of *AtMET1* and *NtMET1* cDNA sequences and the red box marks the 352 bp fragment selected to use as an IR. **b.** the amino acid sequence encoded by the *AtMET1* and *NtMET1* cDNA sequences shown in **a.** Red highlights the conserved DNA methyltransferase motif sequences. The red box marks the region encoded by the 352 bp fragment described in **a.**

6.2.3.2 *Construction of the* 35S*::*AtMET1*-RNAi and* 35S*::*NtMET1*-RNAi constructs*

The 352 bp *AtMET1* fragment was amplified from *Arabidopsis* WT leaf cDNA (Accession Col-0) and the 352 bp *NtMET1* fragment was amplified from tobacco WT leaf cDNA by PCR using primers AtMET1.2F & AtMET1.2R and NtMET1.2F & NtMET1.2R respectively. The *AtMET1* and *NtMET1* PCR fragments were ligated into the pGEMT to produce *AtMET1*-RNAi-pGEMT and *NtMET1*-RNAi-pGEMT respectively (**Figure 6.8a** and **b**). Following transformation into *E. coli*, plasmid from a positive colony for each construct was sequenced.

The sequence of the *AtMET1* fragment was identical to that of the published *AtMET1* sequence (TAIR, 2007). In contrast, seven base differences were detected between the sequence of the *NtMET1* fragment and published sequences of *NtMET1* cDNA (NCBI, 2007. Accessions: AY567977, AB280788 & AB030726). It was deemed unlikely that PCR would induce such a high frequency of errors. However, as tobacco is an allotetraploid the sequence differences could reflect polymorphisms between the *NtMET1* genes derived from the two ancestral species - *N. sylvestis* and *N. tomentosiformis*. To test this, the *NtMET1* fragment was amplified from WT tobacco DNA by PCR and sequenced. Polymorphisms were again present at the same seven nucleotide positions previously identified in the *NtMET1* fragment amplified from cDNA. Consequently, it was concluded this fragment represented a faithful copy which was therefore used in the construction of the *35S*::*NtMET1*-RNAi gene.

The *AtMET1* and *NtMET1* fragments were cut from pGEMT by *Asc*1 and *Swa*1 and ligated in the sense-orientation into *Asc*1- and *Swa*1-cut pFGC5491 to produce *AtMET1*-RNAi-(sense)-pFGC5491 and *NtMET1*-RNAi-(sense)-pFGC5491 constructs respectively. Subsequently a second *AtMET1* and *NtMET1* fragment was cut from pGEMT by *Xma*1 and *BamH*1 and was ligated in the antisense-orientation into *Xma*1- and *BamH*1-cut *AtMET1*-RNAi-(sense)-pFGC5491 and *NtMET1*-RNAi-(sense)-pFGC5491 to produce *AtMET1*-RNAi-(sense & antisense)-pFGC5491 (*35S*::*AtMET1*-RNAi) and *NtMET1*-RNAi-(sense & antisense)-pFGC5491 (*35S*::*NtMET1*-RNAi) constructs respectively (**Figure 6.8c** and **d**).

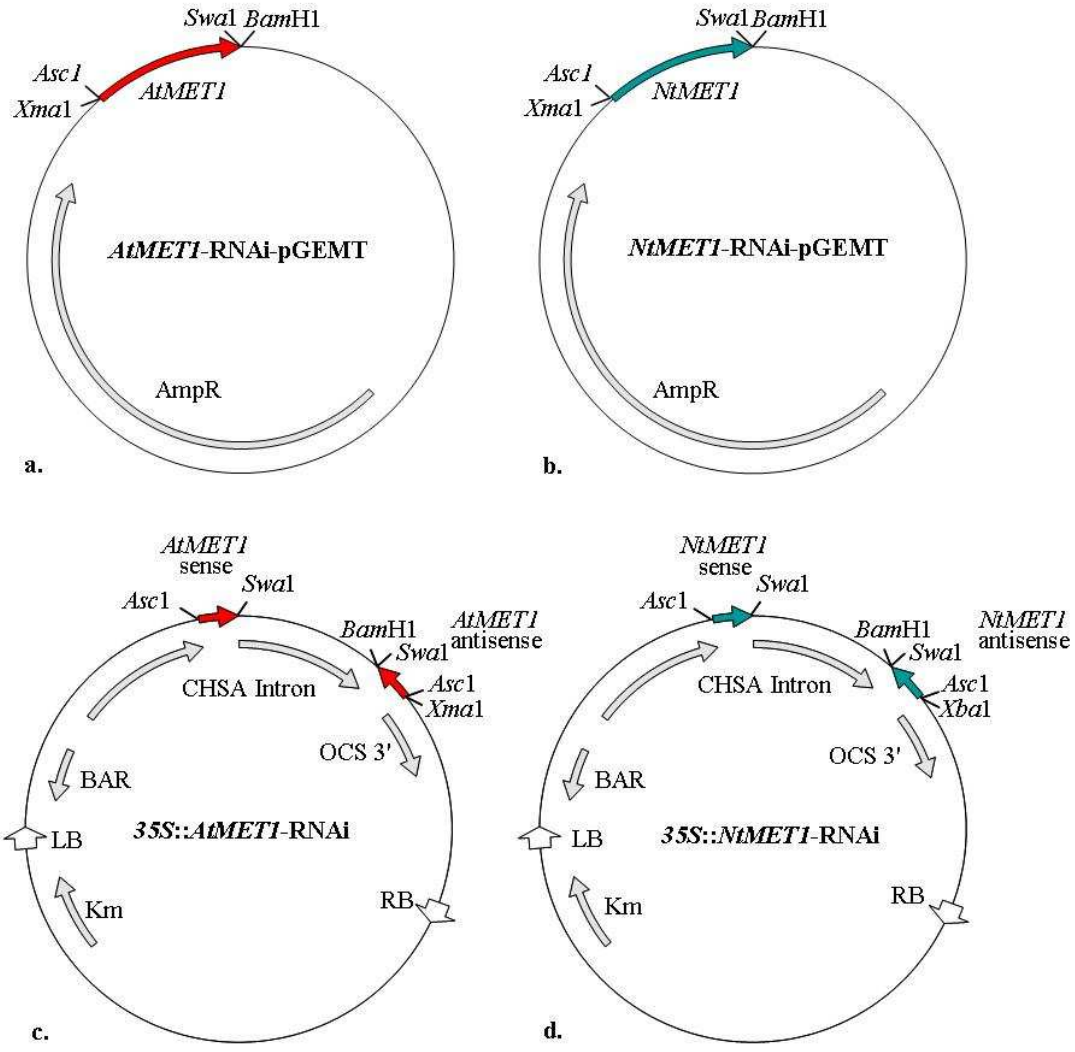


Figure 6.8 Plasmid maps of *AtMET1*-pGEMT (a), *NtMET1*-pGEMT (b), *35S*::*AtMET1*-RNAi (c) and *35S*::*NtMET1*-RNAi (d).

6.2.3.3 *Transformation of the* 35S*::*AtMET1*-RNAi and* 35S*::*NtMET1*-RNAi transgenes into tobacco*

35S::*AtMET1*-RNAi and *35S*::*NtMET1*-RNAi constructs were transformed into *A. tumefaciens* and subsequently the transgenes were transformed into tobacco by leaf disk transformation. Transgenic plantlets were selected on BASTA containing growth media. 17 *35S*::*AtMET1*-RNAi transgenic plantlets were recovered and transferred to soil. However, the majority of apparently PPT resistant *35S*::*NtMET1*-RNAi shoots either died or failed to form roots. Consequently, only three *35S*::*NtMET1*-RNAi transgenic plantlets were recovered and transferred to soil. However, all plantlets transferred to soil were successfully grown to maturity.

6.2.3.4 *Preliminary analysis of* 35S*::*AtMET*-RNAi and* 35S*::*NtMET1*-RNAi lines*

T1 *35S*::*AtMET1*-RNAi and *35S*::*NtMET1*-RNAi lines displayed no obvious phenotype abnormalities indicative of hypomethylation. The methylation level of *35S*::*AtMET1*-RNAi line 1 was analysed using Southern hybridisation as described above (Section **6.2.2.3**). Figure **6.6** (Section **6.2.2.3**) shows that DNA from *35S*::*AtMET1*-RNAi line 1 is equally digested by the methylation-sensitive enzyme *Hap*II and the methylation-insensitive enzyme *Msp*I, indicating that this line is hypomethylated.

Seed was collected from all *35S*::*AtMET1*-RNAi and *35S*::*NtMET1*-RNAi lines and *35S*::*AtMET1*-RNAi and *35S*::*NtMET1*-RNAi lines 1-3 were selected for further analysis in the T2 generation. T2 seedlings were geminated on soil and treated with BASTA. BASTA resistant seedlings were recovered from *35S*::*AtMET1*-RNAi lines 2 and 3 and *35S*::*NtMET1*-RNA lines 1, 2 and 3. However, plants from *35S*::*AtMET1*-RNAi line 1 failed to develop beyond the seedling stage.

T2 plants from *35S*::*AtMET1*-RNAi line 2 were pale and late flowering but had a WT-like floral phenotype. In contrast, T2 plants from *35S*::*AtMET1*-RNAi line 3 and *35S*::*NtMET1*-RNAi lines 1-3 had a phenotype indistinguishable to that of WT lines (Date not shown).

6.2.3.5 *Transgene detection in* 35S*::*AtMET*-RNAi and* 35S*::*NtMET1*-RNAi lines*

PCR was used to check for the presence of transgenes in T2 plants from *35S*::*AtMET1*-RNAi lines 2 and 3 and *35S*::*NtMET1*-RNAi lines 1, 2 and 3. Sequences spanning the *AtMET1* sense and antisense fragments of the *35S*::*AtMET1*-RNAi transgene were

successfully amplified from plants of *35S*::*AtMET1*-RNAi lines 2 and 3, indicating that the transgene within these lines is intact and putatively functional (**Figure 6.9**).

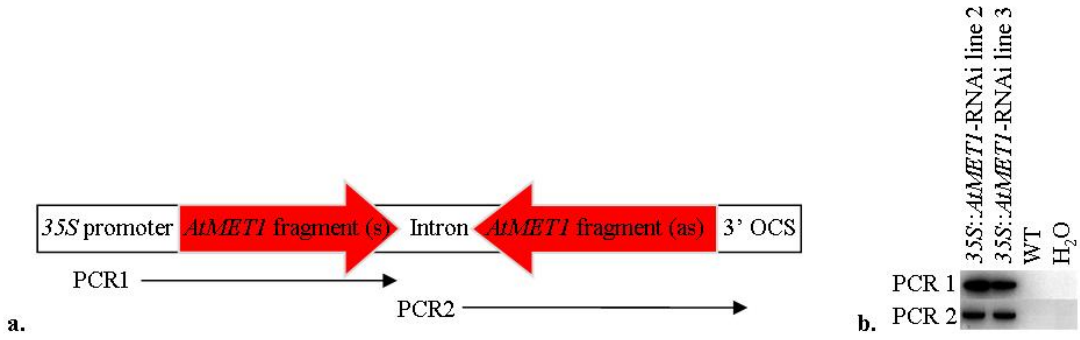


Figure 6.9 Transgene detection in *35S*::*AtMET1*-RNAi lines 2 and 3. a. A schematic of the *AtMET1*-RNAi transgene with arrows indicating the regions amplified by PCR1 and PCR2. **b.** Bands successfully amplified from representative plants of *35S*::*AtMET1*-RNAi lines 2 and 3 using PCR 1 and PCR 2.

A sequence spanning the *NtMET1* sense fragment of the *35*::*NtMET1*-RNAi transgene was successfully amplified from plants of *35S*::*NtMET1*-RNAi lines 1, 2 and 3. However, attempts to amplify the *NtMET1* antisense fragment were unsuccessful. To investigate this further, five PCRs were carried out to amplify overlapping fragments spanning the entire *35S*::*NtMET1*-RNAi transgene from plants of *35S*::*NtMET1*-RNA lines 1, 2 and 3 (**Figure 6.10**). All regions of the transgene were successfully amplified from a sample of the *35S*::*NtMET1*-RNAi plasmid DNA used to transform *A. tumefaciens* and from plasmid DNA extracted from the *A. tumefaciens* colonies used to transform tobacco. However, only the 5' region of the transgene was amplified from plants of *35S*::*NtMET1*-RNAi lines 1, 2 and 3. These findings indicate the *35S*::*NtMET1*-RNAi construct used for the tobacco transformation is intact and putatively functional but is fragmented in *35S*::*NtMET1*-RNAi lines 1, 2 and 3.

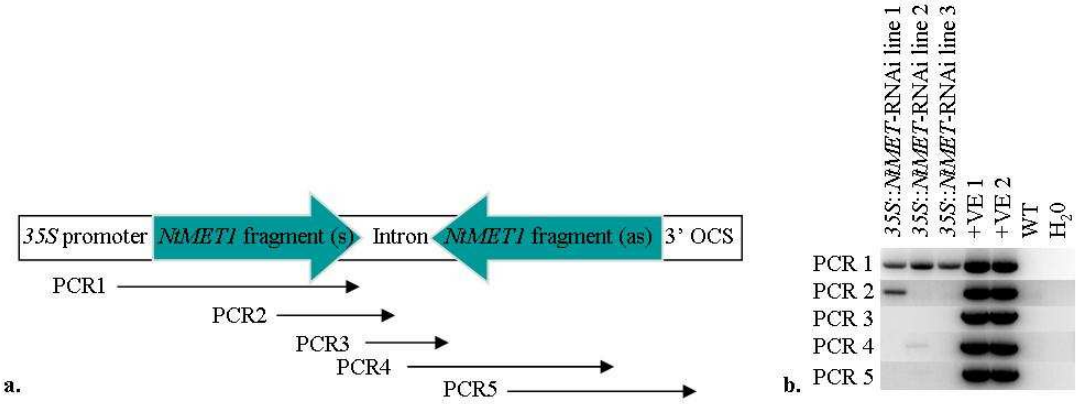


Figure 6.10 Transgene detection in *35S*::*NtMET1*-RNAi lines 1, 2 and 3. a. A schematic of the *35S*::*NtMET1*-RNAi transgene with arrows indicating the regions amplified by PCRs 1-5. **b.** Regions successfully amplified from representative plants of *35S*::*NtMET1*-RNAi lines 1, 2 and 3 using PCRs 1-5. The +VE 1 lane shows fragments amplified from *35S*::*NtMET1*-RNAi the plasmid DNA used to transform *A. tumafciens* and the +VE 2 lane shows fragments amplified from plasmid DNA extracted from the *A. tumafciens* colonies used to transform tobacco

6.3 *Discussion*

The aim of the work described in this chapter was to determine whether the parent-of-origin-specific inheritance of hypomethylated genomes can be used to manipulate seed size in tobacco and to investigate whether a transgene designed to suppress *AtMET1* could be used to induce *NtMET1* suppression. This section discusses the effect of hypomethylation on seed set, viability and development in tobacco. Additionally this section considers the effect of RNAi-induced *NtMET1* suppression on plant regeneration from tissue culture and briefly discusses the potential of using parent-of-origin-specific hypomethylation as a method to manipulate seed size in tobacco. Finally, the effect of *AtMET1* antisense and inverted-repeat transgenes on *NtMET1* expression is briefly considered.

6.3.1 *Parent-of-origin-specific effects of hypomethylated genomes on seed development in tobacco*

In *Arabidopsis*, the inheritance of a hypomethylated genome has a parent-of-origin-specific effect on seed development which phenocopies the effects of interploidy crosses (Adams, S. et al. 2000). [WT x *MET1*as] seed has a maternal-excess phenotype characterised by reduced endosperm proliferation and a low mature seed weight. Conversely, [*MET1*as X WT] seed has a paternal-excess phenotype characterised by endosperm over-proliferation and a high mature seed weight. These findings are consistent with the model suggesting

that hypomethylation of the paternally-inherited genome results in the released silencing of paternally-inherited endosperm-inhibiting genes which would normally be expressed exclusively from the maternally-inherited genome. Conversely, hypomethylation of maternally-inherited genomes results in the released silencing of maternally-inherited endosperm-inhibiting genes which would normally be exclusively paternally-expressed (Adams, S. et al. 2000).

In tobacco, the inheritance of hypomethylated genomes also has striking parent-of-origin-specific effects on seed development. In *35S*::*NtMET1*as X WT crosses the majority of fertilized ovules initiate development but subsequently abort. In contrast, in the reciprocal WT X *35S*::*NtMET1*as cross, the abortion level is low. These parent-of-origin-specific effects closely resemble those described previously for interploidy crosses between diploid (C) and tissue culture cell-derived tetraploid lines (TC) in tobacco; only 2% of seed from C X TC (2x X 4x) crosses were viable and the remaining seed was apoembryonic (lacking an obvious embryo) (Contolini, C. S. and Hughes, W. 1989). In contrast, no apoembryonic seeds were reportedly produced from TC X C (4x X 2X) crosses. Similarly to the finding in *Arabidopsis,* these results show that the outcome of reciprocal *35S*::*NtMET1*as X WT crosses, phenocopies the outcome of reciprocal interploidy crosses.

The inheritance of a hypomethylated genome also has a parent-of-origin-specific effect on mature seed size in tobacco. 'Large' seed from the *35S*::*NtMET1*as X WT cross was heavier than that from the WT (+) cross. Conversely, 'large' seed from the WT X *35S*::*NtMET1*as cross is lighter than that from the WT (+) cross. These findings are consistent with the model suggesting that hypomethylation of the paternally-inherited genome results in the relaxed silencing of endosperm-inhibiting genes and visa versa for hypomethylation of the maternally-inherited genome (Adams, S. et al. 2000). These results also suggest that *NtMET1* has a homologous role to *AtMET1* in silencing imprinted genes in tobacco.

In *Arabidopsis*, the number of seeds produced per pod is negatively correlated with the average seed weight (Adams, S. 2002). Consequently, it could be argued that the heavy weight of seed produced from the *35S*::*NtMET1*as X WT cross is the result of low seed set per pod, as opposed to the over-expression of endosperm-promoting genes. However, a similarly low seed set was produced from the *35S*::*NtMET1*as (+) cross and yet the weight

of seed produced from these crosses was less than that produced from the WT (+) cross (**Figure 6.4**). Consequently, reduced seed set does not explain the high seed weight from the *35S*::*NtMET1*as X WT cross.

Indeed, it is possible that the high seed abortion from *35S*::*NtMET1*as X WT crosses results from excessive over-expression of endosperm-promoting genes i.e. the effect of paternal excess. *Arabidopsis* is unusual in that viable seed is produced from reciprocal diploid X tetraploid interploidy crosses; thus seed can tolerate both maternal and paternal excess. However, in many plant species, reciprocal diploid X tetraploid interploidy crosses are unsuccessful revealing widespread intolerance of both maternal and paternal excess (Haig, D. and Westoby, M. 1991). In general, maternal excess is tolerated to a greater degree than paternal-excess; often seed with maternal excess is smaller than normal but plump, well formed and viable, whereas seed with paternal excess is often normal-sized but shrivelled and non-viable (Haig, D. and Westoby, M. 1991). Seed abortion from paternal excess is associated with endosperm over-proliferation. For example, in *Arabidopsis*, seed abortion from 2x X 6x and *MET1*as X 4x crosses is correlated with an increased number of peripheral endosperm nuclei and overgrowth of the chalazal endosperm and nodules (Adams, S. et al. 2000, Scott, R. J. et al. 1998). Additionally, seed abortion of *fis*-class mutants is associated with mass endosperm over-proliferation (Kiyosue, T. et al. 1999, Scott, R. J. et al. 1998, Sørensen, M. B. et al. 2001, Vinkenoog, R. et al. 2000). It is not known how excessive endosperm over-proliferation causes seed abortion, but failure of endosperm to cellularise could prevent conduction of maternal resources to the embryo. Significantly, endosperms produced by extreme maternal excess, although small, are fully cellularised.

In tobacco, the high level of seed abortion from C X TC (2x X 4x) and *35S*::*NtMET1*as X WT crosses and the apparent low level of abortion from the reciprocal cross indicates that maternal excess is tolerated more than paternal excess. However, since the rate of endosperm proliferation in the seed from C X TC (2x X 4x) and *35S*::*NtMET1*as X WT crosses was not measured these results should be considered preliminary and the proposal that seed abortion is associated with, or caused by endosperm over-proliferation, must await confirmation. Nevertheless, the heavy weight of viable seed from the *35S*::*NtMET1*as X WT cross provides strong evidence for this.

6.3.2 *Sporophytic and gametophytic effects of hypomethylation on seed size and set*

Above (Section **6.3.1**), it is suggested that the patent-of-origin specific effects of hypomethylation on seed size and set in tobacco are the results of altered imprinting. An alternative possibility is that these changes in seed development result from sporophytic or gametophytic parent-of-origin specific effects of hypomethylation. For example, on the male side, hypomethylation could affect anthers or sperm development. It is noteworthy that tobacco sperm are dimorphic (Tian, H. Q. et al. 2001) and the generative cell of the tobacco male gametophyte is reportedly less methylated than the vegetative cell (Oakeley, E. J. et al 1997). It is therefore possible that controlled methylation fluctuations are important in sperm development. Consequently, the low weight of [WT X *35S*::*NtMET1*as] seed could, in some part, be due to the effects of hypomethylation on sperm development or the stability or expressivity of either one of the two paternally transmitted genomes.

Similarly, hypomethylation could also have a maternal sporophytic effect on seed size in tobacco. The *sin1* mutant exemplifies the involvement of maternal sporophytic tissue in seed development. A *sin1* homozygous mutant embryo develops normally when nursed by a *sin1* heterozygous seed parent but abnormally when nursed by a *sin1* homozygous seed parent (Ray, S. et al. 1996). Thus a functional maternal sporophytic copy of *SIN1* is essential for normal seed development. It is possible that hypomethylation caused by *NtMET1*as expression results in the mis-regulation of maternal sporophytic genes which are essential for seed development.

When *35S*::*NtMET1*as plants are used as seed parents in crosses with either WT or *35S*::*NtMET1*as pollen parents, approximately 50 % less seed was set compared with the WT (+) cross. However, in the reciprocal cross there was no observable reduction in seed set. Therefore, hypomethylation caused by *NtMET1*as expression appears to have a maternal effect on seed set. This could infer a sporophytic effect on carpel development or a gametophytic effect on ovule development. The floral phenotype of *35S*::*NtMET1*as plants supports the former hypothesis. Two floral mutations caused by hypomethylation in *Arabidopsis* result from the mis-regulation of floral regulatory genes. The first causes an increased number of anthers and carpels and is correlated with hypermethylation and silencing of the floral developmental regulator *SUPERMAN* (Jacobsen, S. E. and Meyerowitz, Elliot M. 1997). The second causes an *agamous*-like flower, where stamens are converted to petals and the ovary to a new internal flower. This phenotype is correlated

with the hypermethylation and silencing of the floral regulator *AG* (Jacobsen, S. E. et al. 2000). It is possible that hypomethylation also causes the mis-regulation of floral regulatory genes in tobacco and that this has a knock-on effect resulting in reduced fertility. Alternatively, hypomethylation could cause a gametophytic effect resulting in abnormal female gametophyte development or the disruption of pollen tube signaling or reception. However, it should be noted that these hypotheses do not explain why reciprocal *35S*::*NtMET1*as and WT crosses phenocopy interploidy crosses.

6.3.3 *The effect of* 35S*::*NtMET1-*RNA expression on tobacco regeneration from tissue culture*

The *35S*::*NtMET1*-RNAi lines recovered from leaf disk transformation, have a fragmented and presumably non-functional copy of the *35S*::*NtMET1*-RNAi transgene. The ability to regenerate plants from leaf disks explants in tissue culture is likely to depend on an intricate regulation of gene expression. One possible explanation for the inability to recover *35S*::*NtMET1*-RNAi lines with a full length and functional transgene is that transgene expression causes the mis-regulation of genes essential for plant regeneration. In *Arabidopsis*, *MET1* is required to regulate the expression of the mitogen-activated protein kinase *YODA* and the homeodomain transcription factors *WUSCHEL related homeobox 2* and *8*, which are responsible for cell fate specification during embryogenesis (Xiao, W. et al. 2006b). Additionally, *MET1* is required for the establishment of auxin gradients during embryogenesis and to regulate expression of the auxin efflux carrier *PIN-formed 1* (Xiao, W. et al. 2006b). It is likely that plant regeneration in tobacco requires similar genes and it is possible that the mis-regulation of these genes inhibits plant regeneration.

However, hypomethylated *35S*::*NtMET1*as lines were successfully regenerated from leaf disk explants in tissue culture (Nakano, Y. et al. 2000), indicating that a level of hypomethylation during plant regeneration can be tolerated. The *35S*::*NtMET1*-RNAi construct could induce a higher level of hypomethylation which can not be tolerated. Alternatively, it is possible that the hypomethylation induced by the *35S*::*NtMET1*-RNAi construct only reduces the likelihood of achieving plant regeneration and that 35S::NtMET1-RNAi lines with a full length transgene could be produced if the transformation was repeated on a larger scale.

6.3.4 *Altering seed development in tobacco using parent-of-origin-specific hypomethylation*

The complementary seed size phenotypes produced from reciprocal crosses between *35S*::*NtMET1*as and WT plants in tobacco highlights a potential to manipulate seed size in tobacco by the parent-of-origin-specific inheritance of hypomethylated genomes. However, other findings described here highlight potential limitations of this approach. Firstly, the data suggests that seed development in tobacco tolerates only weak paternal excess and therefore a limited amount of endosperm over-proliferation (Section **6.3.1**). This is likely to limit the extent to which seed size can be increased, unless the factors causing the intolerance can be identified and attenuated. Secondly, hypomethylation has a maternal effect which causes a reduction in seed set (Section **6.3.2**). A reduced seed set is likely to counteract any benefits of increasing seed size. However, it is possible that the impact on seed set will be reduced if hypomethylation is tissue-specific. And thirdly plant regeneration from tissue-culture is apparently prevented by hypomethylation (Section **6.3.3**). This may pose a problem for the generation of transgenic lines but again this could be circumvented using tissue-specific hypomethylation.

6.3.5 *The effect of transgenes designed to suppress AtMET1 on methylation in tobacco*

Due to time constraints only preliminary methylation and phenotype analysis of *35S*::*AtMET1*as and *35S*::*AtMET1*-RNAi lines was performed. Southern hybridisation detected hypomethylation in a number of *35S*::*AtMET1*.F1.as, *35S*::*AtMET1*.F2.as and *35S*::*AtMET1*-RNAi lines. This indicates that the expression of *AtMET1* antisense or inverted-repeat fragments encoding conserved DNA methyltransferase motifs can induce *NtMET1* suppression. However, hypomethylation can be induced by the process of plant regeneration from tissue culture. For example, in maize, 39 % of families derived from tissue culture were found to be hypomethylated at CCGG sites (Kaeppler, S. M. and Phillips, R. L. 1993). Therefore, it is possible that the hypomethylation detected in a number of the *35S*::*AtMET1*as and *35S*::*AtMET1*-RNAi lines could have been induced by the transformation process and not by transgene *per se*. To confirm that hypomethylation is induced by transgene activity it will be necessary to demonstrate that the hypomethylation is associated with *NtMET1* suppression.

No phenotype abnormalities indicative of hypomethylation were detected in the T1 generation of *35S*::*AtMET1*as and *35S*::*AtMET1*-RNAi lines. However, abnormalities were

observed in T2 *35S*::*AtMET1*-RNAi lines. Further analysis is needed to determine whether or not these abnormalities are caused by *NtMET1* suppression and hypomethylation. Additionally, it is still necessary to investigate whether or not seed size is altered in *35S*::*AtMET1*as and *35S*::*AtMET1*-RNAi lines.

Chapter 7 - *Parent-of-origin-specific effects of hypomethylation on sporophytic development*

7.1 *Introduction*

In mammals, genomic imprinting i.e. monoallelic expression, occurs during embryogenesis *and* postnatal development (Refer to section **1.3.2**). To achieve this, parent-of-origin-specific epigenetic modifications that set during gametogenesis and maintained after fertilisation and throughout development (Ueda, T. et al. 1992).

In contrast, in plants, imprinting has only been reported in the endosperm (Refer to section **1.3.3**). As the endosperm is a determinate tissue, parent-of-origin-specific epigenetic modifications inherited by the endosperm are not transmitted to the next generation. Genes that are imprinted in the endosperm can be biallelically expressed in the embryo (Kinoshita, T. et al. 1999). Reportedly, transcription of some paternally-inherited alleles is delayed in the embryo after fertilisation (Vielle-Calzada, J. P. et al. 2000). This indicates that there may be parent-of-origin-specific epigenetic modifications at some loci immediately after fertilization. However, such genes were found to be biallelically expressed by 3-4 DAP (Vielle-Calzada, J. P. et al. 2000) suggesting that these are not maintained. In support of this suggestion, a thorough literature search found no examples of parent-of-origin-specific expression or epigenetic difference outside the endosperm.

However, preliminary observations indicated phenotypic differences between *met1-9* heterozygotes produced from reciprocal crosses between *met1-9* homozygous and WT plants; *met1-9* heterozygotes derived from a *met1-9* homozygous seed parent ([*met1-9* x WT] plants) appeared less delayed in their development and were architecturally different from *met1-9* heterozygotes derived from a *met1-9* homozygous pollen parent ([WT x *met1-9*] plants). These differences are surprising given that [*met1-9* x WT] and [WT x *met1-9*] plants are genetically identical having inherited one normally methylated genome and one hypomethylated genome. If parentally-inherited genomes are epigenetically equivalent, it is expected that the phenotype of the *met1-9* heterozygotes would be near-identical irrespective of the origin of the hypomethylated genome. Preliminary observations to the contrary indicate that parental genomes could be epigenetically distinct. The aim of the work described in this chapter was to investigate this parent-of-origin-specific

phenomenon by validating these preliminary observations and determining what causes phenotype differences between reciprocal *met1-9* heterozygotes.

7.2 *Results*

7.2.1 *Phenotype comparison of [*met1-9 *x WT] and [WT x* met1-9*] heterozygotes*

Preliminary observations indicated differences in the rate of development and architecture of [*met1-9* x WT] and [WT x *met1-9*] heterozygotes. Such phenotype differences can arise if plants are differentially affected in their transition from the vegetative to the reproductive growth phases. This transition requires the detection and response to environmental and endogenous floral cues and results in a change in identity of the lateral primordia produced by the shoot apical meristem (SAM) (Araki, T. 2001). In *Arabidopsis*, during the vegetative phase, the SAM produces leaf primordia to form the rosette. In response to floral cues, stem elongation is initiated and the SAM acquires inflorescence meristem identity. During the early reproductive phase, the SAM produces auxiliary inflorescence meristems subtended by cauline leaves primordia. Finally, during the late reproductive phase the SAM produces determinate floral primordia. To validate the preliminary observations of phenotypic differences between [*met1-9* x WT] and [WT x *met1-9*] plants, the phenotype of these plants was compared during the vegetative and reproductive growth phases.

7.2.1.1 *Phenotype analysis of [*met1-9 *x WT] and [WT x* met1-9*] plants*

The developing vegetative rosettes of reciprocal *met1-9* heterozygotes were visually similar. Both produced much larger and glossier rosette leaves than WT plants and had broader and rounder rosettes. However, [WT x *met1-9*] plants produced significantly more rosette leaves than [*met1-9* x WT] plants (T=7.26, P=0.000, d.f.=21) and flowered significantly later (T=4.2, P=0.000, d.f.=20) (**Figure 7.1a and b**). In contrast, [*met1-9* x WT] plants produced significantly more auxiliary flowering shoots than [WT x *met1-9*] plants (T=-3.16, P=0.005, d.f.=18) and consequently developed a bushier architecture (**Figure 7.1c**). There was no difference in the height of [*met1-9* x WT] and [WT x *met1-9*] plants when flowering was initiated and the height of both *met1-9* heterozygotes remained comparable relative to the number of flowers produced from the primary inflorescence stem (**Figure 7.1d**). However, the primary inflorescence stem of [WT x *met1-9*] plants flowered significantly longer than that of [*met1-9* x WT] plants (T=-2.68, P=0.016, d.f.=17), produced more flowers than [*met1-9* x WT] plants, although not significantly

more, and reached a significantly greater height than [*met1-9* x WT] plants (W=63, N_1=9 ,N_2=10 P=0.03) (**Figure 7.1e, f and g**). Both *met1-9* heterozygotes produced more cauline leaves from auxiliary flowering shoots and secondary inflorescence stems than WT plants, yet neither *met1-9* heterozygote displayed obvious floral defects or fertility abnormalities (**Figure 7.2**). Despite many common characteristics, [*met1-9* x WT] and [WT x *met1-9*] plants were nevertheless easily distinguishable from the initiation of the reproductive growth phase onwards; in general, [WT x *met1-9*] plants had a more *met1-9*-like phenotype, whereas [*met1-9* x WT] plants had a more WT-like phenotype (**Figure 7.2**).

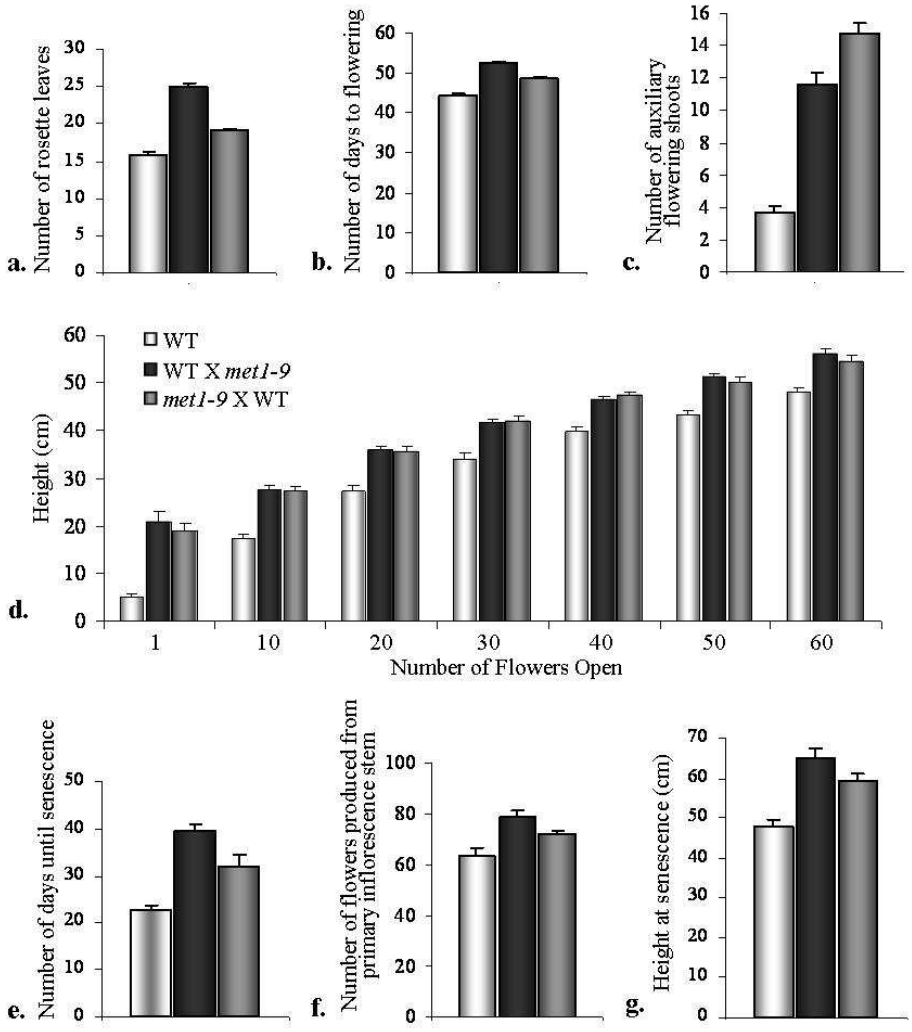


Figure 7.1 Phenotype comparison of [*met-9* x WT] and [WT x *met1-9*] plants. For all graphs the error bars show standard error.

Figure 7.2 Phenotype comparison of [*met1-9* x WT] and [WT x *met1-9*] heterozygotes post-bolting. Arrows point to examples of the increased number of cauline leaves observed on the auxiliary flowering shoots and secondary inflorescence stems of the reciprocal *met1-9* heterozygotes. Scale bar, 2 cm.

7.2.1.2 *Transition of [*met1-9 *x WT] and [WT x* met1-9*] plants from vegetative to reproductive growth*

Using the data described above **Figure 7.3** was constructed to illustrate how reciprocal *met1*-9 heterozygotes differ in their transition from vegetative to reproductive growth. In both *met1-9* heterozygotes, all phases of development are prolonged to different extents. [WT x *met1-9*] have a longer vegetative phase than [*met1-9* x WT] plants which suggests they are more delayed in achieving inflorescence meristem identity. In contrast, [*met1-9* x WT] plants have a longer early reproductive phase then [WT x *met1-9*] plants. However, [WT x *met1-9*] plants are later flowering than [WT x *met1-9*] plants. In summary, [*met1-9* x WT] and [WT x *met1-9*] plants have differentially prolonged vegetative and early reproductive phases and overall [WT x *met1-9*] plants are more delayed in their initiation of flowering than [*met1-9* x WT] plants.

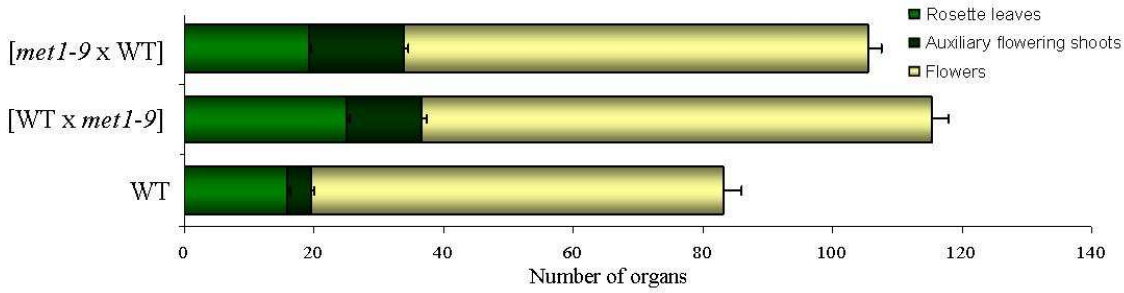


Figure 7.3 A comparison of the number of rosette leaves, auxiliary flowering shoots and flowers produced by the shoot apical meristem of [*met1-9* x WT] and [WT x *met1-9*] heterozygotes.

7.2.2 *Investigating a possible association between ectopic* FWA *expression and the differential flowering time of* met1-9 *heterozygotes*

Late flowering is a characteristic of hypomethylation and associated with ectopic *FWA* expression. The late-flowering trait of hypomethylated *ddm1* mutants was genetically mapped to a chromosomal region containing *FWA* (Kakutani, T. 1997) and the late flowering trait of *fwa-1* gain-of-function mutants is correlated with ectopic *FWA* expression and hypomethylation of direct repeats 5' to the gene (Soppe, W. J. J. et al. 2000). Targeted remethylation of these repeats in a *ddm1*-induced late-flowering background is sufficient to silence ectopic expression of *FWA* and correct flowering time (Kinoshita, Y. et al. 2007). These findings strongly suggest that the late flowering phenotype of hypomethylated mutants is caused by ectopic *FWA* expression and in accordance with this, the late-flowering phenotype of hypomethylated *met1-9* mutants and *35s*::*MET1*-RNAi lines is correlated with ectopic *FWA* expression (Chapter 3, section **3.2.4.2** and Chapter 4 section **4.2.5.5**). The late-flowering phenotype of *ddm1*, *met1-9* and *fwa* gain-of-function mutants have more rosettes leaves and auxiliary flowering shoots than WT plants, suggesting that FWA expression causes both these traits (Chapter 3 section 3.2.5.2, Kakutani, T. 1997, Koornneef, M et al. 1991).

In light of the correlation between hypomethylation, ectopic FWA expression and late flowering it was hypothesised that the differential late flowering phenotype of reciprocal *met1*-9 heterozygotes is caused by ectopic *FWA* expression and the inheritance of hypomethylated genomes in a parent-of-origin-specific manner.

To test this hypothesis, reciprocal *met1*-9 heterozygotes were assayed for ectopic FWA expression. Additionally, the phenotype of [*met1-9fwa-3* x WT] and [WT x *met1-9fwa-3*] heterozygous double loss-of-function mutants was compared. If the phenotype differences between reciprocal *met1*-9 heterozygotes are caused by the parent-of-origin-specific ectopic FWA expression, it is expected that these differences will be eliminated in reciprocal *met1-9fwa-3* heterozygotes, as these plants will not inherit a functional and hypomethylated *FWA* allele.

7.2.2.1 *Ectopic* FWA *expression in [met1-9 x WT] and [WT x* met1-9*] heterozygotes*

The *FWA*::*GFP* reporter was used to determine whether *FWA* is ectopically expressed in [*met1-9* x WT] and [WT x *met1-9*] heterozygotes. *met1-9* homozygotes carrying the *FWA*::*GFP* reporter were reciprocally crossed with WT plants to produce [*met1-9 FWA*::*GFP* x WT] and [WT x *met1-9 FWA*::*GFP*] plants. These plants were germinated on plant growth media and seedlings were analyzed for GFP at 4 days after germination. Strong *GFP* expression was observed throughout [*met1-9 FWA*::*GFP* x WT] and [WT x *met1-9 FWA*::*GFP*] seedlings indicating that FWA is ectopically expressed in both *met1-9* heterozygotes (**Figure 7.4**).

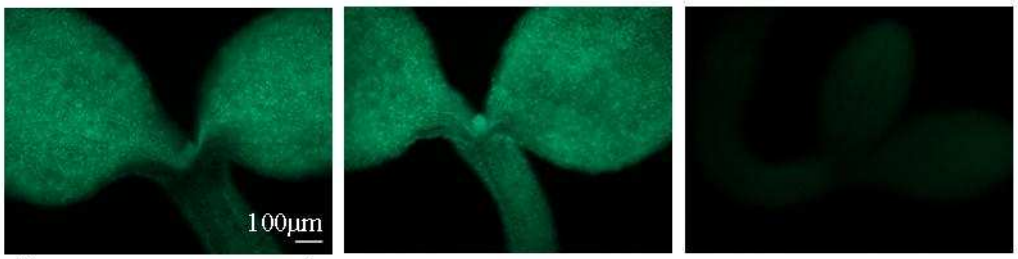


Figure 7.4 *FWA*::*GFP* expression in [*met1-9 FWA*::*GFP* x WT] and [WT x *met1-9 FWA*::*GFP*] seedlings.

To validate this result, RT-PCR was performed on RNA extracted from the rosette leaves of [*met1-9* x WT] and [WT x *met1-9*] plants (**Figure 7.5**). FWA transcript was detected in both met1-9 heterozygotes again suggesting that FWA is ectopically expressed in these plants.

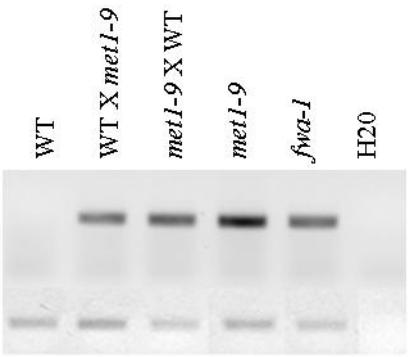


Figure 7.5. RT PCR testing for ectopic *FWA* expression in [*met1-9* x WT] and [WT x *met1-9*] heterozygotes.

7.2.2.2 *Phenotype comparison of [*met1-9fwa-3 *x WT] and [WT x* met1-9fwa-3*] plants*

The phenotype of [*met1-9fwa-3* x WT] and [WT x *met1-9fwa-3*] plants was analysed to determine whether the inheritance of a hypomethylated *FWA* allele is responsible for the differential flowering time delay of reciprocal *met1-9* heterozygotes.

The *fwa-3* loss-of-function mutant is a T-DNA insertion line of the *Arabidopsis* accession Wassileskija (WS). To make *met1-9fwa-3* double homozygous, homozygous *fwa-3* plants were crossed with homozygous *met-9* plants. Double homozygous mutants were identified in the F2 population by genotyping using PCR. It should be noted that as the *met1-9* T-DNA insertion is in the Col-0 background and the *fwa-3* T-DNA insertion is in the WS background, each *met1-9fwa-3* double mutant is likely to have a genetically different Col-0/WS hybrid background.

met-9fwa-3 homozygotes displayed some phenotype abnormalities characteristic of *met1-9* homozygotes, for example they had reduced stature and severely reduced fertility. However, unlike *met1-9* homozygotes, *met-9fwa-3* homozygotes were early flowering and produced few rosette leaves or auxiliary flowering shoots. This strongly suggests that the late flowering phenotype of *met1-9* mutants is caused by the ectopic expression of *FWA*.

To produce reciprocal *met-9fwa-3* heterozygotes, *met-9fwa-3* homozygous mutants were reciprocally crossed with WT (Col-0) plants. As a consequence of the severely reduced fertility of the *met-9fwa-3* homozygotes, different plants were used as the pollen and seed parents to achieve useful levels of seed set from theses crosses. Consequently [*met1-9fwa-3* x WT] and [WT x *met1-9fwa-3*] plants may have different Col-0/WS hybrid genetic

backgrounds and thus the phenotype comparison of these plants should be considered as preliminary at this stage.

The phenotype of reciprocal *met1-9fwa-3* heterozygotes was compared at various stages of their development. In contrast to reciprocal *met1-9* heterozygotes, reciprocal *met1-9fwa-3* heterozygotes did not produce a significantly different number of rosette leaves (W=145, N_1=13, N_2=13, P=0.124). On average [WT x *met1-9fwa-3*] plants produced less than one rosette leaf more than [*met1-9fwa-3* x WT] plants and [*met1-9fwa-3* x WT] plants produced less than one auxiliary shoot more than [WT x *met1-9fwa-3*] plants (**Figures 7.6a** and **b**). It should also be noted that the number of rosette leaves produced by the reciprocal *met1-9fwa-3* heterozygotes was intermediate to that produced by WT (Col-0) and WT (WS) plants (**Figure 7.6a**).

At later stages of development phenotype differences between reciprocal *met1-9fwa-3* heterozygotes were more apparent. Analysis of plants at 5 weeks old revealed that the primary inflorescence stem of [*met1-9fwa-3* x WT] plants reached a greater height and produced more flowers than that of [WT x *met1-9fwa-3*] plants (**Figure 7.6c** and **d**). However it should be noted that the phenotype of both *met1-9fwa-3* heterozygotes was still intermediate to that of WT (Col-0) and WT (WS) plants (**Figure 7.7a**).

More strikingly, approximately 50% (6/13) of [WT x *met1-9fwa-3*] plants had severely reduced fertility whereas [*met1-9fwa-3* x WT] plants were fully fertile (n=13) (**Figure 7.7b**). Reduced fertility was neither observed in either WT (Col-0) plants or WT (WS) plants. Examination of flowers from the [WT x *met1-9fwa-3*] plants with reduced fertility revealed that anther dehiscence had failed to occur in the majority of flowers (**Figure 7.7c**) Occasionally anther dehiscence did occur and seed set was achieved indicating that the plants were not completely sterile.

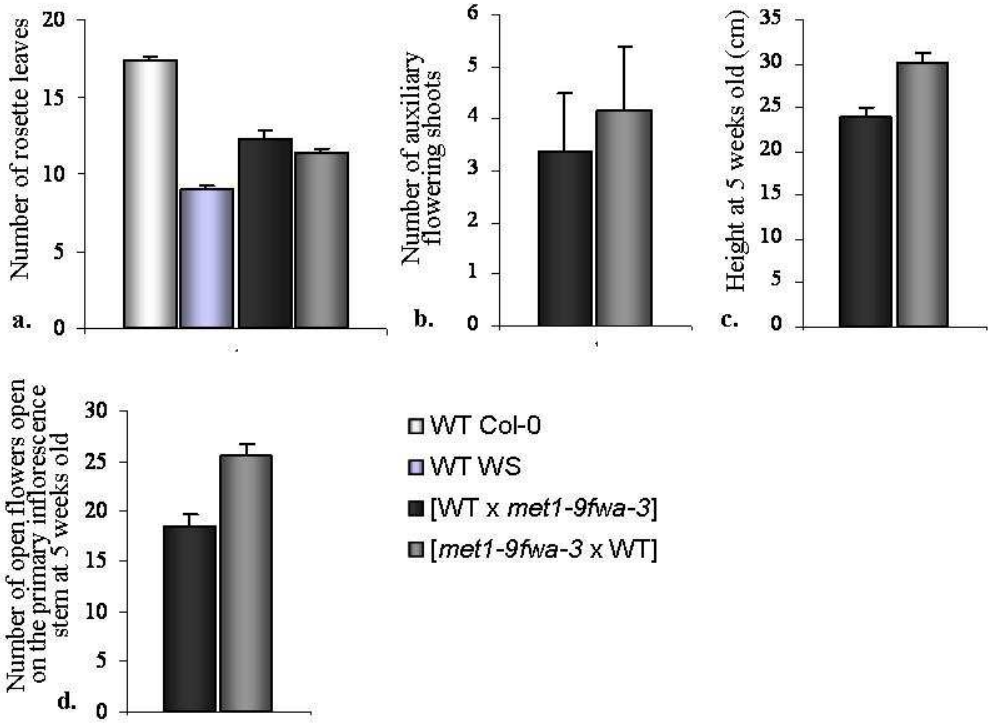


Figure 7.6 Phenotype comparison of [*met-9fwa-3* x WT] and [WT x *met1-9fwa-3*] plants. For all graphs the error bars show standard error.

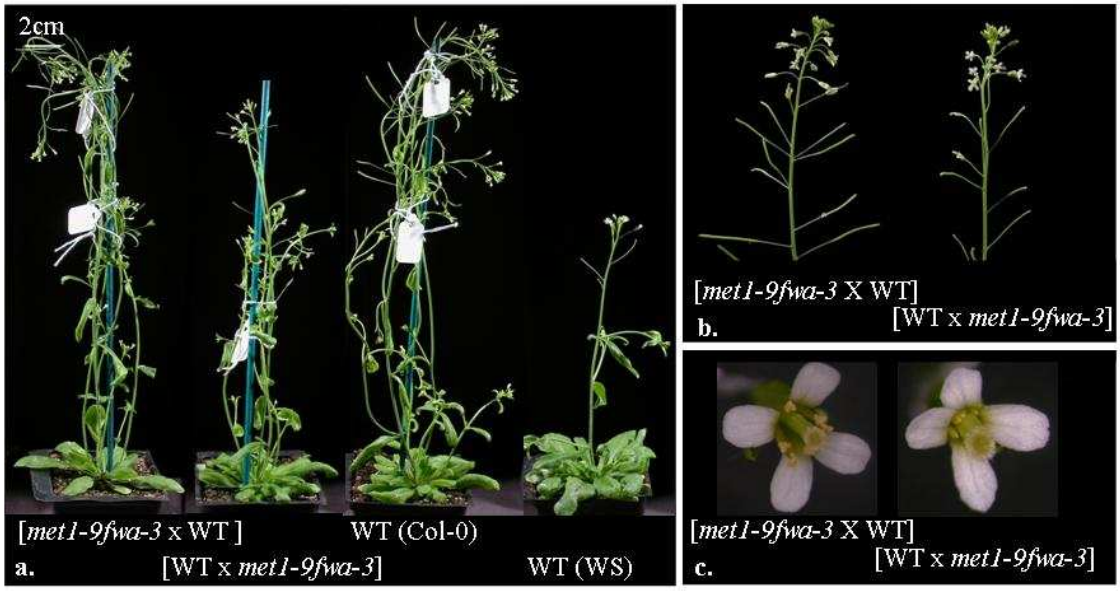


Figure 7.7 Phenotype comparison of [*met1-9fwa-3* x WT] and [WT x *met1-9fwa-3*] heterozygotes post-bolting. a. Photograph of plants at 5 weeks old illustrating that the phenotype of [*met1-9fwa-3* x WT] and [WT x *met1-9fwa-3*] plants is intermediate to that of WT (Col-0) and WT (WS) plants**. b.** Photograph of the primary inflorescence stems showing the reduced fertility observed in approximately 50 % of [WT x *met1-9fwa-3*] plants. **c.** Photograph of flowers showing the failure of anther dehiscence in approximately 50 % of [*met1-9fwa-3* x WT] plants.

7.3 *Discussion*

The aim of the work described in this chapter was to validate preliminary observations that [*met1-9* x WT] and [WT x *met1-9*] plants are phenotypically distinct and to investigate the cause of phenotype differences. It has been demonstrated that reciprocal *met1-9* heterozygotes are differentially delayed in their transition from the vegetative to the reproductive growth phases and that this is primarily caused by ectopic expression of *FWA*. In this section phenotype difference between reciprocal *met1-9* heterozygotes are reviewed and hypotheses explaining how ectopic expression of *FWA* could cause such phenotype differences are proposed. Finally, consideration is made to the implication of these results with respect to parent-of-origin-specific epigenetic inheritance.

7.3.1 *Phenotypic differences between [*met1-9 *x WT] and [WT x* met1-9*] plants*

In agreement with preliminary observations, reciprocal *met1-9* heterozygotes are phenotypically distinct. [WT x *met1-9*] plants produce more rosette leaves than [*met1-9* x WT] plants which suggests they are more delayed in detecting or responding to floral cues and achieving inflorescence meristem identity. Additionally, [WT x *met1-9*] plants are later-flowering than [*met1-9* x WT] plants, indicating that are also more delayed in achieving the floral meristem identity.

In apparent contrast to this trend, [*met1-9* x WT] plants produce more auxiliary flowering shoots than [WT x *met1-9*] plants; this gives [*met1-9* x WT] plants a bushier appearance. Auxiliary inflorescence meristems, which give rise to auxiliary flowering shoots, can either arise from the basipetal (top to bottom) differentiation of pre-existing leaf primordia at the same time as floral meristems are generated on the flanks of primary the inflorescence (Hempel, F. D. and Feldman, L. J. 1994) or they can be generated on the flanks of the primary inflorescence meristem prior to the generation of floral meristems (Suh, S. et al. 2003). The former process defines a single phase transition to flowering and the latter defines a two-step phase transition to flowering (Suh, S. et al. 2003). If plants undergo a single-phase transition to flowering, an increased production of auxiliary flowering shoots could indicate that an increase in the number of pre-existing leaf primordia that have been differentiated. Alternatively, where plants undertake a two-phase transition to flowering, the increased production of auxiliary flowering shoots could indicate a delay in achieving floral meristem identity. Depending on the mode of floral transition [*met1-9* x WT] plants

could either have an increased ability to differentiate pre-existing leaf primordia or be more delayed in achieving floral meristem identity compared with [WT x *met1-9*] plants.

It was also observed that [WT x *met1-9*] plants flower for longer than [*met1-9* x WT] plants. It is possible that the longevity of plants is correlated with flowering time and the extent of vegetative development. Examples in *Arabidopsis* indicate that early-flowering lines tend to have a shorter life than those which are late-flowering. For example, lines over-expressing the flowering time gene *FLOWERING LOCUS T* (FT) have little vegetative structure and terminate flowering earlier (Teper-Bamnolker, P. and Samach, A. 2005), whereas the extremely late-flowering *ft apetala1* (*ap1*) double mutants have extensive vegetative structure and can live for more than six months (Ruiz-Garcia, L. et al. 1997). It is possible that [WT x *met1-9*] plants flower for longer than [*met1-9* x WT] plants because the prolonged phase of vegetative development provides the additional structure and strength needed to transport resources further and aid the development of more siliques.

Arabidopsis plants can tolerate a 50 % reduction in MET1 activity without suffering any phenotype abnormalities (Kankel, W. et al. 2003). However, once the genome has been exposed to mutants that induce hypomethylated, remethylation is extremely slow despite the presence of WT alleles (Finnegan, E. J. et al. 1996, Genger, R. K et al. 1999, Kakutani, T. et al. 1999). For these reasons it is suggest that the phenotype differences between reciprocal *met1-9* heterozygotes are not due to the parent-of-origin specific inheritance of the *met1-9* allele *per se* but instead due to the parent-of-origin specific inheritance of hypomethylated genomes. This hypothesis could be tested by analysing the phenotype of plants produced from reciprocal crosses between *met1-9* homozygotes and a MET1 over-expression line; these plants will inherit parent-of-origin-specific hypomethylated genomes but should not be deficient in MET1 activity.

7.3.2 *Parent-of-origin-specific ectopic expression of* FWA

As argued above, phenotypic differences between reciprocal *met1-9* heterozygotes are believed to constitute parent-of-origin-specific effects caused by the inheritance of a hypomethylated genome. It was also hypothesized that the differential flowering time of reciprocal *met1-9* heterozygotes is caused by parent-of-origin-specific ectopic expression of *FWA*. Ectopic *FWA* expression was detected in both *met1-9* heterozygotes indicating

that the late flowering phenotype of these plants is correlated with ectopic *FWA* expression. Moreover, the flowering time differences between reciprocal *met1-9* heterozygotes was greatly reduced between reciprocal *met1-9fwa-3* heterozygotes indicates that inheritance of a hypomethylated *FWA* is largely responsible the parent-of-origin-specific effects on flowering.

It is likely that ectopic FWA delays flowering by inhibiting the flowering time protein FT. The *ftfwa* (*FT* loss-of-function, *FWA* gain-of-function) double mutant has the same phenotype as both the *ft* and *fwa* single mutants (Koornneef, M. et al. 1998). Additionally, both *ft* or *fwa* and *ap1* double mutants have similar phenotypes (Ruiz-Garcia, L. et al. 1997). Thus, consistent with the model that FWA inhibits FT, mutant phenotype analysis indicates that ectopic expression of *FWA* has a similar effect as loss of *FT* expression. Moreover, FWA interacts with FT in a yeast-hybrid assay highlighting a possible mechanism by which FWA could directly inhibit FT activity (Ikeda, Y. et al. 2007).

FT is a floral integrator gene. Floral integrators receive signals from multiple environmental and endogenous flowering time pathways and regulate the expression of floral meristem identity genes accordingly. *FT* is a key floral integrator because it receives signals from multiple flowering time pathways including vernalization, long day, autonomous flowering, light quality, gibberellin and temperature-dependent pathways (Bernier, G. and Perilleux, C. 2005). Consequently, the action of FT is likely to be complex. Expression analysis has revealed that the *FT*-dependent transition to flowering involves the up- or down-regulation of hundreds of genes within the shoot apical meristem (Schmid, M. et al. 2003). In the literature, FT is commonly referenced with respect to its involvement in activating the floral meristem identity gene *AP1*; using double mutant phenotype analysis it was demonstrated that FT acts redundantly with LEAFY to activate *AP1* (Ruiz-Garcia, L. et al. 1997). However, it is likely that FT also acts to initiate inflorescence meristem identity as *ft* mutants have an increased number of rosette leaves *and* auxiliary flowering shoots (Ruiz-Garcia, L. et al. 1997). Additionally it is reported that *FT* expression regulates the transcript accumulation of flowering time genes *FRUITFULL* and *SEPALLATA3* within rosette leaves and it is suggested that FT may play a role in controlling the differentiation of leaf primordia during the transition to flowering (Teper-Bamnolker, P. and Samach, A. 2005).

As ectopic expression of *FWA* delay flowering and [WT x *met1-9*] heterozygotes are later flowering than [*met1-9* x WT] heterozygotes, it is possible that *FWA* is expressed at a higher level when inherited from a paternally-hypomethylated background from a maternally-hypomethylated background. The model shown in **Figure 7.8** illustrates that a high level of *FWA* expression in [WT x *met1-9*] plants could result in strong inhibition of FT and a long delay in flowering. In contrast, a relatively low level of *FWA* expression in [*met1-9* x WT] plants could result in weaker inhibition of FT and a shorter delay in flowering.

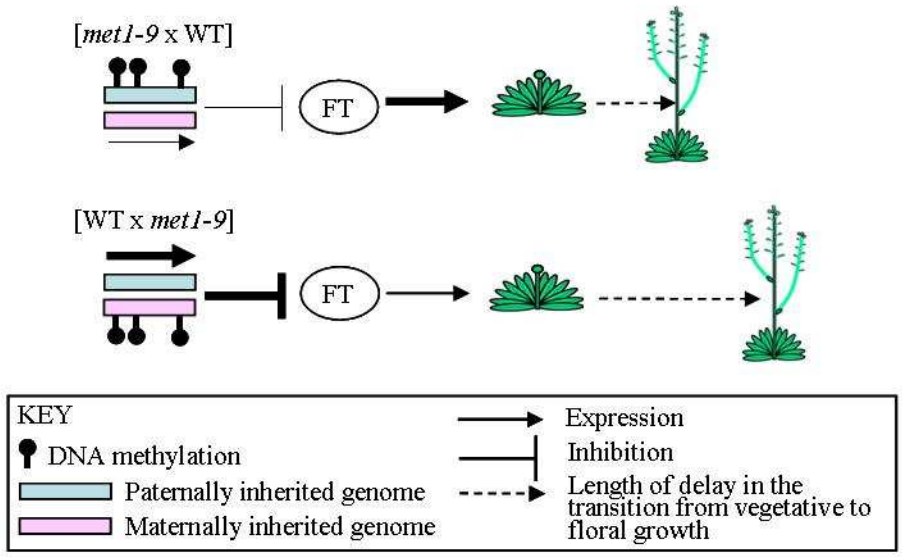


Figure 7.8 Model suggesting how the parent-of-origin-specific ectopic expression of *FWA* could cause the differential delay in flowering of reciprocal *met1-9* heterozygotes. The widths of the arrows are positively correlated with the predicted level of expression and similarly the widths of the inhibition bars are positively correlated with the predicted level of inhibition.

This model could explain why [WT x *met1-9*] plants produce more rosette leaves and are later-flowering than [*met1-9* x WT] plants but it may not explain why [*met1*-9 x WT] plants produce more auxiliary flowering shoots than [WT x *met1-9*] plants. Microscopic analysis of the shoot apical meristem of *fwa* gain-of-function mutants revealed *fwa* mutants undergo two-step phase transition to flowering (Suh, S. et al. 2003), i.e. secondary inflorescence meristems are generated on the flanks of the primary inflorescence meristem prior to the generation of floral meristems. Therefore, it is possible, that despite achieving inflorescence meristem identity more quickly than [WT x *met1-9*] plants, [*met1-9* x WT]

plants are more delayed in detecting or responding to cues that trigger the production of floral meristems compared with [WT x *met1-9*] plants.

As FT functions to activate floral meristem identity genes it is possible that FT is inhibited more between achieving inflorescence meristem and floral meristem identity in [*met1-9* x WT] plants compared with [WT x *met1-9*] plants. It is possible that this could occur if *FWA* is ectopically expressed in a parent-of-origin specific manner at different levels during different phases of development. *FWA* may be ectopically expressed more strongly during vegetative development when inherited from a paternally-hypomethylated parent but during the early reproductive phase when inherited from a maternally-hypomethylated parent. This switch in parent-of-origin specific expression levels could coincide which the dramatic reorganization that the shoot apical meristem must incur during the transitions from the vegetative to reproductive growth phase.

However, it may be unjustified to assume that a difference in the amount of auxiliary flowering shoots infers a differential ability to achieve floral meristem identity without considering other factors such as the age of the plants. [WT x *met1-9*] plants are older than [*met1-9* x WT] plants when they achieve inflorescence meristem identity, and therefore they may achieve floral meristem identity more quickly because they have been exposed to floral cues for longer. A comparison of the relative length of the early reproductive phase, i.e. the number of auxiliary flowering shoots produced, may be useful to evaluate architectural difference between plants, but because floral signalling is complex and not clearly understood, there may be inherent problems with using this as a parameter to compare the rate of phase transition during flowering.

The hypothesis that *FWA* is expressed in a parent-of-origin-specific manner when inherited from a hypomethylated background could be tested by analyzing the level of ectopic *FWA* expression in reciprocal *met1-9* heterozygotes at various stages of development. Semi-quantitative RT PCR performed on rosette leaves detected no difference in *FWA* transcript levels in reciprocal *met1-9* heterozygotes; however this analysis may not have been sensitive enough or performed at an appropriate stage of development. It may be more appropriate to analyze *FWA* expression in the shoot apical meristem as FWA is likely to have the greatest impact on flowering in this tissue.

7.3.3 *Putative parent-of-origin-specific epigenetic modifications of the* FWA *locus*

It is hypothesized that *FWA* is ectopically expressed in a parent-of-origin-specific manner when inherited from a hypomethylated background. As ectopic *FWA* expression results from hypomethylation, it is possible that the *FWA* locus is differentially methylated when maternally- or paternally inherited from a hypomethylated background. This section considers how differences in methylation of the *FWA* locus from reciprocal *met1-9* heterozygotes could arise.

Expression of *FWA*, whether ectopic or not, is associated with the hypomethylation of direct repeats 5' to the *FWA* transcriptional start (Soppe, W. J. J. et al. 2000). Bisulphite sequencing of *FWA* in the endosperm revealed that the imprinted expression of *FWA* is associated with a dramatic reduction of methylation at CpG sites, and a slight reduction of methylation at CpNpG sites and asymmetric sequences, of *FWA* direct repeats (Kinoshita, T. et al. 2004). Similarly, bisulphite sequencing revealed that vegetative ectopic expression of FWA in *ddm1*-induced late flowering mutants is also correlated with loss of methylation of *FWA* direct repeats primarily at CpG sites (Kinoshita, Y. et al. 2007). It is therefore assumed that the ectopic vegetative expression of *FWA* in *met1-9* homozygous mutants (Chapter 3, section **3.2.4.2**) is also caused by the loss of *MET1*-dependent CpG methylation at the same sites. In *met1-9* homozygotes, the direct repeats of *FWA* alleles entering male and female gametogenesis are likely to be fully hypomethylated at CpG sites as they have passaged through multiple cell divisions in the absence of MET1. If *FWA* alleles are differentially methylated when maternally- or paternally-inherited from *met1-9* homozygotes, they must receive differential re-methylated. Two models suggesting how parent-of-origin specific remethylation could occur are described below.

Firstly, it is possible that the *FWA* locus is differentially remethylated during the male and female gametophyte generation. As the gametophytes of *met1-9* homozygotes do not posse a functional *MET1* allele, such remethylation must be independent of MET1. It is possible that the *FWA* inherited from a paternally-hypomethylated background is remethylated less than that inherited from a maternally-hypomethylated background because the male gametophyte generation is comparatively shorter than the female gametophyte generation. Differential remethylation of the *FWA* locus during gametogenesis would result in the differential methylation of the *FWA* locus in male and female gametes. This model could therefore be tested using bisulphite sequencing to determine gamete-specific methylation at

the *FWA* locus. Locus specific methylation analysis in gametes has been achieved in maize (Gutierrez-Marcos, J. F. et al. 2006) but may be technically difficult in *Arabidopsis*.

Alternatively, the *FWA* locus may be differentially 'marked' during the male and female gametophyte generation by an epigenetic modification which results in parent-of-origin-specific differential remethylation after fertilization. This model is illustrated in **Figure 7.9**. Examples of parent-of-origin specific differential remethylation of alleles have been described in mammals (Davis, T. L. et al. 1999, Davis, T. L. et al. 2000, Ueda, T. et al. 2000). Such epigenetic marks must be detected by remethylation machinery after fertilization. The epigenetic mark could be histone methylation or a different form of chromatin modification. If the *FWA* locus is differentially remethylated after fertilization, differential methylation of the *FWA* locus would not be expected in gametes, but parent-of-origin-specific methylation would be expected in young seedlings. This model could therefore also be tested using bisulphite sequencing to compare methylation levels at the *FWA* locus in male and female gametes and parent-of-origin-specific methylation levels at the *FWA* locus in seedlings of reciprocal *met1-9* heterozygotes.

Irrespective of how parent-of-origin-specific methylation is achieved, it is predicted that it must be maintained at least during vegetative development to cause the parent-of-origin-specific affects on develop described for reciprocal *met1-9* heterozygotes.

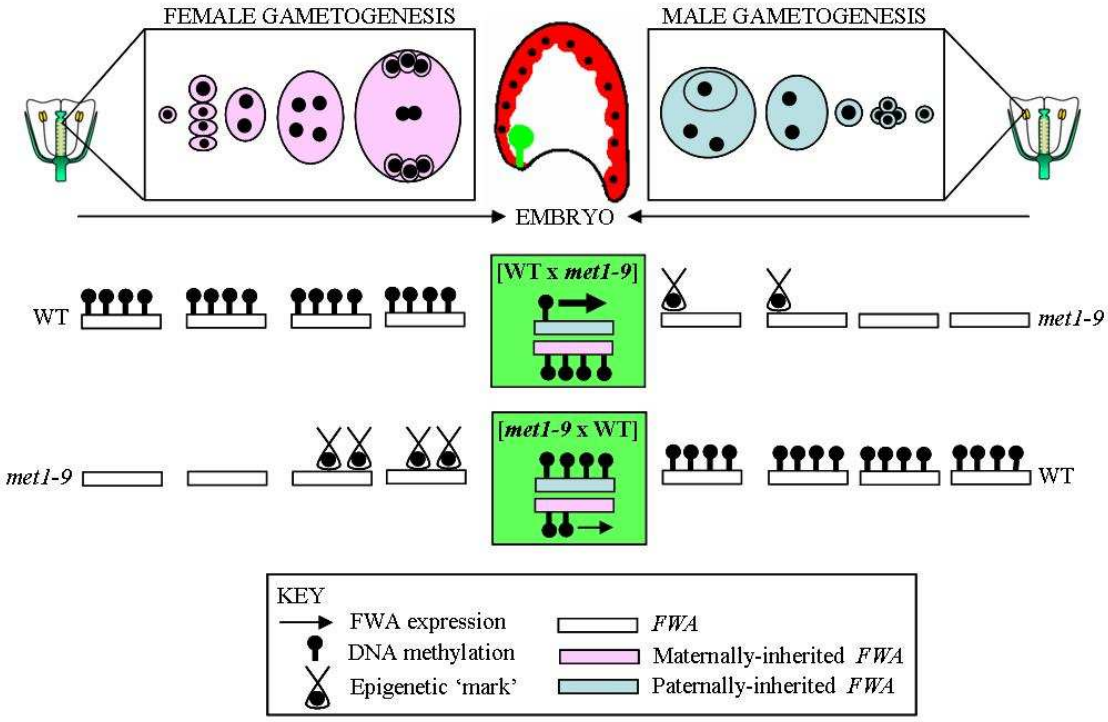


Figure 7.9 Model suggesting how epigenetic 'marks' received during gametogenesis in *met1-9* homozygotes could result in parent-of-origin-specific remethylation after fertilization. The width of the *FWA* expression arrow positively correlates with the predicted level of expression during vegetative development.

7.3.4 *Phenotypic differences between [*met1-9fwa-3 *x WT] and [WT x* met1-9fwa-3*] plants*

In support of the suggestion that flowering time differences between reciprocal *met1*-9 heterozygotes are due to parent-of-origin specific *FWA* expression, it was demonstrated that these flowering time differences are dramatically reduced in reciprocal *met1-9fwa-3* heterozygotes (See section 7.2.2.2). However, phenotype differences between reciprocal *met1-9fwa-3* heterozygotes were still observed, for example, at 5 weeks old [WT x *met1-9fwa-3*] plants were taller than [*met1-9fwa-3* x WT] plants. Before continuing the discussion of these results, it is important to note that phenotype comparison between reciprocal *met1-9fwa-3* heterozygotes is complicated by the likelihood that these plants have different Col-0/WS genetic backgrounds. Potential, but unknown, effects of these different hybrid backgrounds limit the interpretation of these results. Consequently this work should be considered preliminary until this variable can be eliminated; this could be done by repeating the comparison of reciprocal *met1-9fwa-3* heterozygotes using *met1* and *fwa* loss-of-function mutants in the same genetic background.

Nevertheless, it is interested to note that the variation between reciprocal *met1-9fwa-3* heterozygotes was less than and intermediate to that between WT (Col-0) and WT (WS) plants for several traits analyzed (**Figure 7.6a** and **Figure 7.7a**). It is therefore possible that this variation is due to differences in the genetic backgrounds of plants, as opposed to a parent-of-origin-specific effect resulting from inheritance of genomes from an *fwa-3met1-9* homozygote. In accordance with this suggestion, it is hypothesized that phenotype differences between the reciprocal *met1-9fwa-3* heterozygotes will be further reduced in plants with homologous genetically backgrounds.

However, the severely reduced fertility observed in 50 % of [WT x *met1-9fwa-3*] plants is unlikely to be caused by the hybrid Col-0/WS background, as both WT (Col-0) and WS (WS) plants are fully fertile. It is possible that this phenotype is dependent on the ancestry of the *fwa-3* allele (**Figure 7.10**). However, to date, this putative grandparental-effect can not be explained.

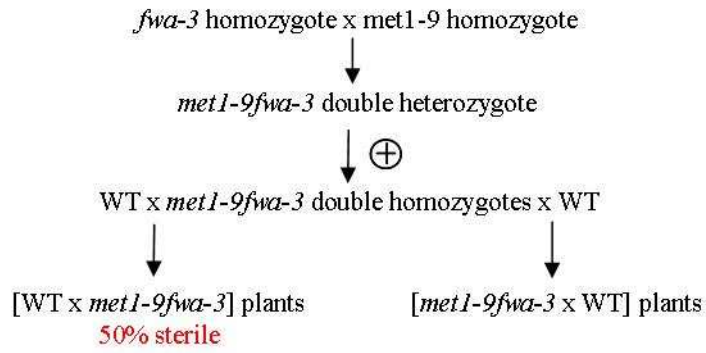


Figure 7.10 Ancestry of [WT x *met1-9fwa-3*] and [*met1-9fwa-3* x WT] plants

7.3.5 *Parent-of-origin-specific epigenetic inheritance outside of the seed*

If expression analysis and bisulphite sequencing confirm the hypotheses that the *FWA* inherited from a hypomethylated background is remethylated and differentially expressed in a parent-of-origin specific manner, this would provide the first evidence of parent-of-origin-specific epigenetic inheritance, and thus genomic imprinting, outside of the seed. It is suggested that the *FWA* locus is differentially remethylated or epigenetically 'marked' during the gametophyte generation that these modifications result in the parent-of-origin-specific expression during vegetative development of the sporophyte. It is possible that other loci may also be similarly modified in a parent-of-origin-specific manner and

additionally it is possible that loci inherited from a normally methylated background could also receive sex-specific epigenetic modifications. No examples of parent-of-origin-effects on sporophytic development have previously been reported. This could indicate that this phenomenon only affects a limited number of loci. However, it is possible that parent-of-origin-effects on sporophytic development have never been considered before and therefore the scale of this phenomenon is difficult to predict. Inheritance of the *FWA* locus from a hypomethylated background provides an excellent model for the study of this phenomenon because flowering time differences resulting from the putative differential remethylation of the *FWA* locus is an easy proxy to monitor. Further work should focus on validating the hypothesis proposed above.

Chapter 8 – *Final discussion and further work*

The primary aims of the work described in this thesis were to achieve a greater understanding of the role that maintenance DNA methyltransferases play in controlling genomic imprinting and to investigate the potential to alter seed size by suppressing their activity. The work described in thesis provides new tools and techniques to further investigate imprinting, as well as new insights in to the role of the MET1 gene family.

8.1 *The role of maintenance DNA methyltransferases in plant genomic imprinting*

This thesis first reported work on putative roles for MET2a, MET2b and MET3 in controlling imprinted gene expression (Chapter 1). The analysis strongly suggests that these genes play no role in imprinting and therefore that MET1 is the sole DNA methyltransferase required to maintain imprinting in plants. This highlights a significant difference from the imprinting system in mammals which is dependent on a number of methyltransferases (Chapter 1, section **1.4.1.2**). It can therefore be concluded that although similar epigenetic modifications are used to achieve parent-of-origin-specific expression in these two taxa, key elements of the process are quite different.

8.2 *DNA methylation-dependent mechanisms of genomic imprinting*

When the work described in this thesis was started, little was known about the tissue-specific requirement of *MET1* for genomic imprinting. More recently, several studies have demonstrated a requirement of *MET1* during the gametophyte generation. For example, the paternal- and maternal-inheritance of a *met1-6* allele from *met1-6* heterozygotes correlates with a decrease and increase in seed size respectively (Xiao, W. et al. 2006a). As *met1* heterozygotes are deficient in *MET1* activity only during the gametophyte generation, these findings suggested that loss of *MET1* activity during the male and female gametophyte generation results in relaxed silencing of paternally contributed endosperm-inhibiting and maternally contributed endosperm-promoting respectively. In accordance with the former suggestion, it was also demonstrated that the paternal-silencing of *FWA* and *FIS2* is lost in the paternally-derived progeny of *met1-3* heterozygotes (Jullien, P. E. et al. 2006b). These findings are consistent with the *MET1*::*GFP* expression profile reported in this thesis, which indicates that *MET1* is expressed throughout the male and female gametophyte generation and is therefore available to maintain the methylation and silencing of imprinted genes throughout this stage of the life cycle (Chapter 4). These findings are also consistent

with other studies which indicate that *MET1* activity during the male and female gametophyte generation is required to maintain the silencing of pericentromeric repeat sequences and to achieve the correct regulation of genes involved in embryogenesis (Saze, H. et al. 2003, Xiao, W. et al. 2006b).

The requirement of *MET1* during the gametophyte generation for the control of imprinted gene expression in the endosperm is consistent with the predictions made by Model 1 proposed in Chapter 1 (Section **1.4.2.6**). This model predicts that the default state of imprinted genes is methylated and that imprinting is achieved by the sex-specific demethylation alleles prior to gametogenesis. The model is dependent on *MET1* activity during the sporophyte generation to maintain the methylation of all imprinted loci, and during the gametophyte generation to maintain the silencing of paternally contributed endosperm-inhibiting genes and maternally contributed endosperm-promoting genes.

To probe the gametophytic and sporophytic role of *MET1,* an approach was developed here to suppress *MET1* tissue-specifically using promoter-specific *MET1*-RNAi constructs. This produced tools that can be used to signal hypomethylation and altered genomic imprinting. Analysis using these tools revealed that tissue-specific expression of a *MET1* IR fragment can induce tissue-specific *MET1* suppression. However, this approach needs refining before it can be used to test the effects of *MET1* suppression on genomic imprinting. Further work should focus on making the modifications proposed in Chapter 5 (Section **5.3.**) and subsequently testing the tissue-specific requirement of *MET1* for genomic imprinting. This would allow further validation of model and enhance our understanding of how MET1 is involved in controlling imprinted gene expression. Additionally this would help evaluate whether tissue-specific *MET1* suppression can be used as an approach to manipulate seed size, as discussed below.

Model 1 is also dependent on mechanisms to achieve the sex specific-demethylation of endosperm-inhibiting and endosperm-promoting genes. The central cell-specific DNA glycosidase DME is suggested to demethylate and activate the expression of the maternally-expressed imprinted genes *FWA, FIS2* and *MEA* (Choi, Y. et al. 2002, Jullien, P. E. et al. 2006b, Kinoshita, T. et al. 2004). More recently it has been demonstrated that ectopic expression of *DME* in stamen and pollen induces the ectopic expression of 94 genes (Ohr, H. et al. 2007). It has not yet been determined whether these genes are

imprinted but they are likely candidates. It is therefore possible that DME plays a central role in the demethylation and activation of a large number of maternally-expressed imprinted genes.

To date, no paternally-expressed imprinted genes that depend on MET1 for imprinting, have been identified. Nevertheless the complementary seed size phenotypes produced from reciprocal crosses between WT and hypomethylated plants provides strong evidence for their existence. The mechanism by which these genes are demethylated is also unknown. One possibility is that these genes are actively demethylated by a DME-like (DML) protein. Recently is has been demonstrated that three DML proteins, REPRESSOR OF SILENCING1, DML2 and DML3, have demethylation activity and actively demethylate at least 179 loci (Morales-Ruiz, T. et al. 2006, Penterman, J. et al. 2007, Zhu, J. et al. 2007). It is possible that DMLs are involved in the demethylation of paternally contributed endosperm-promoting genes. However, since these DML proteins are not expressed tissue-specifically and their demethylation activity appears to have a more prominent role in protecting the genome against deleterious methylation rather than regulating gene expression (Penterman, J. et al. 2007).

An alternative possibility is that paternally contributed endosperm-promoting genes are passively demethylated. Passive demethylation can occur in the absence of *MET1* during several generations of cell division (Saze, H. et al. 2003). The findings presented here suggest that *MET1* is expressed throughout anther development and in the male gametophyte generation indicating that a period of MET1 absence allowing passive demethylation is unlikely. However, one model suggests that passive demethylation can occur if DNA binding proteins deny DNA methyltransferases access to newly replicated DNA (Bird, A. 2002). The feasibility of this mechanism has been verified using an artificially methylated episome containing EBNA1 or lac repressor binding sites (Hsieh, C. L. 1999, Lin, I. G. et al. 2000). It is possible therefore that DNA binding proteins are involved in the demethylation of endosperm-promoting genes.

In apparent support of the above hypothesis, analysis of *MET1*::*GFP* expression analysis during the male gametophyte generation suggested that *MET1* is expressed at a low level in microspore mother cells and mono- and bi-nucleate pollen (Chapter **4.3.1.1**). These results indicate that there may be a period of reduced *MET1* activity during this stage of

development, possibly because MET1 is denied access to DNA. Moreover, in tobacco, it is suggested that the generative cell of the male gametophyte generation undergoes a phase of hypomethylation (Oakeley, E. J. et al. 1997). Both of these findings need to be validated. In order to do this, further work should focus on analysing methylation dynamics during the gametophyte generation. This could be done using bisulphite sequencing to monitor the levels of methylation at selected loci before meiosis and after each mitotic division in the gametophyte generation. However, it is likely that the mechanisms controlling the expression of endosperm-promoting genes will not be resolved until such genes are identified. Nevertheless, it is concluded that Model 1 provides the most comprehensive representation of our understanding of DNA methylation-dependent genomic imprinting to date.

8.3 *The potential to manipulate seed size by altering genomic imprinting*

In Chapter 1 (section **1.5**), it was suggested that seed size could be altered by suppressing *MET1* tissue-specifically during anther or carpel development and thus releasing the silencing of genes to be paternally- or maternally-contributed endosperm-promoting and –inhibiting genes respectively. This thesis reports one an approach to achieve this in *Arabidopsis* – by driving a *MET1* IR fragment from tissue-specific promoters. The analysis of promoter-specific *MET1*-RNAi lines indicated that *MET1* suppression and hypomethylation is not induced in the same developmental window in which tissue-specific promoters are capable of driving expression. However, the detection of hypomethylation in pollen from *AP3*::*MET1*-RNAi lines indicates that tissue-specific *MET1* suppression and hypomethylation can be induced using this approach which could therefore be used to alter seed size if refined appropriately. Such refinements are outlined in Chapter 5 (section **5.3.3**) and further work should focus on testing them.

This thesis also analysed the effects of parent-of-origin-specific hypomethylation on the seed development in tobacco. The analysis indicates that the maternal- and paternal-inheritance of hypomethylated genomes has a paternalising and maternalising effect on seed development respectively. This indicates that the effects of parent-of-origin-specific hypomethylation are conserved in divergent plants species. An approach to manipulate seed size by altering parent-of-origin-specific methylation could therefore be effective in a wide variety of species, including cereals. However, the apparent paternalising and maternalising effects on seed development from reciprocal *35S*::*NtMET1*as X WT crosses

still needs to be confirmed by cytological analysis of endosperm proliferation. Further work should aim to do this and thus test the suggestion that seed abortion from the *35S*::*NtMET1*as X WT cross is caused by paternal excess.

Intolerance to paternal excess, which is suggested to cause seed abortion from the *35S*::*NtMET1*as X WT cross in tobacco, could significantly hinder the ability of the proposed approach to increase seed size. The weak inbreeder/strong outbreeder (WISO) hypothesis suggests that parental conflict, and thus the parental 'genetic strength' conveyed by imprinting, will be less intense in self-pollinating species than in outbreeders (Brandvain, Y. et al. 2005). If the 'genetic strength' of genomes from inbreeders is less than that of outbreeders, it is possible that inbreeders may tolerate excess of their own parental genomes, or parent-of-origin-specific hypomethylation, more than outbreeders. In agreement with this, the viability of seed from reciprocal diploid X tetraploid crosses in *Arabidopsis* has been ascribed to the reduced parental conflict in this predominantly self-fertilizing species (Scott, R. J. et al. 1998). Most cereal species are predominantly self-fertilizing suggesting that they may also be capable of tolerating a degree of maternal/paternal excess and therefore a degree of parent-of-origin-specific hypomethylation. However, tobacco is also predominantly an inbreeding species but is nevertheless apparently intolerant of paternal excess/maternal hypomethylation.

One possible approach to attenuate intolerance to paternal excess/hypomethylation is to decrease the level of hypomethylation induced, and thus the extent to which imprinting is altered. The approach could be tested in tobacco by pollinating the *35S*::*NtMET1*as lines, which reportedly have varying degrees of hypomethylation (Nakano, Y. et al. 2000), with WT pollen and subsequently testing for a correlation between the level of abortion and the level of hypomethylation. A positive correlation would indicate that the problem of seed abortion caused by the maternal-inheritance of hypomethylated genomes could be overcome by reducing the level of hypomethylation

Additionally, this thesis investigated that potential of inducing hypomethylation in one plant species, namely tobacco, using a transgene targeted against the conserved DNA-methyltransferase motifs of a second species, namely *Arabidopsis*. Further work is needed to complete the analysis of *35S*::*AtMET1*as and *35S*::*AtMET1*-RNAi tobacco lines but preliminary analysis indicates that this approach is feasible. This would enable the parent-of-origin-specific effects of hypomethylated on seed development to be analysed in a broad

spectrum of species without the need to identify and clone the major maintenance DNA methyltransferases.

It is concluded that the potential to manipulate seed development by parent-of-origin-specific hypomethylation remains strong but further work needed is to develop this approach. It will also be necessarily to evaluate the expediency of this approach over others.

8.4. *Imprinting in the sporophyte generation*

In Chapter 7, it was proposed that the *FWA* locus may be remethylated and expressed in a parent-of-origin-specific manner during the vegetative phase of sporophytic development when inherited from a *met1-9* homozygote. It was further suggested that other loci could be subject to similar parent-of-origin-specific modifications and that loci inherited from a WT background could also be modified in a parent-of-origin-specific manner. It is therefore possible that imprinting could occur outside of the seed.

In agreement with these hypotheses, a putative parent-of-origin-specific enrichment of histone H3 lysine 27 trimethylation (H3K27m3) at 5s rDNA gene arrays has since been reported in sporophytic cells of *met1-3* and *ddm1-5* homozygous mutants (Mathieu, O. et al. 2005). It was found that H3K27m3 is redistributed from euchromatin to heterochromatin in *met1-3* and *ddm1-5* homozygotes. Using a combination of immunostaining using an antibody highly specific to H3K27m3 and DNA fluorescence *in situ* hybridisation, it was revealed that 5s rDNA gene arrays in *met1-3* and *ddm1-5* homozygotes are enriched with H3K27m3 at only one of the two possible loci available in the diploid genome. This finding indicates that hypomethylated genomes may retained some epigenetic memory of their parent-of-origin that can direct further parent-of-origin-specific epigenetic modifications.

The putative occurrence of imprinting outside of the seed could greatly impact on current theories as to why imprinting evolved and what mechanisms control imprinting in plants. For these reasons, priority for further work should given to testing the hypotheses discussed above and in Chapter 7 (section **7.3**) using the approaches proposed in that chapter (section **7.3**).

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